METHODS FOR IDENTIFYING SUBJECTS AT RISK OF MELANOMA AND TREATMENTS THEREOF

Related Patent Applications

[0001] This patent application claims the benefit of provisional patent application 60/410,595 filed September 11, 2002 and provisional patent application 60/422,344 filed October 29, 2002, having attorney docket number 524593038000 and 524593038001, respectively. Each of these provisional patent applications names Richard B. Roth *et al.* as inventors. Each of these provisional patent applications is hereby incorporated herein by reference in its entirety, including all drawings and cited documents.

Field of the Invention

[0002] The invention relates to genetic methods for identifying predisposition to melanoma and treatments that specifically target the disease.

Background

[0003] In some parts of the world, especially among western countries, the number of people who develop melanoma is increasing faster than any other cancer. In the United States, for example, the number of new cases of melanoma has more than doubled in the past twenty years. The probability of developing melanoma increases with age, but this disease effects people of all age groups. Melanoma is one of the most common cancers in young adults.

[0004] Melanoma occurs when melanocytes (pigment cells) become malignant. Most pigment cells are in skin, and when melanoma begins its etiology in the skin it is referred to as coetaneous melanoma. Melanoma may also occur in the eye and is called ocular melanoma or intraocular melanoma. Rarely, melanoma arises in the meninges, the digestive tract, lymph nodes or other areas where melanocytes are found. Within the skin, melanocytes are located throughout the lower part of the epidermis, the latter being the surface layer of the skin. Melanocytes produce melanin, which is the pigment that gives skin its natural color. When skin is exposed to the sun, melanocytes produce more pigment, causing the skin to tan or darken.

[0005] Sometimes, clusters of melanocytes and surrounding tissue form benign growths referred to as moles or nevi (singular form is nevus). Cells in or near the nevi can divide without control or order and form malignant tumors. When melanoma spreads, cancer cells often are found in the lymph nodes. If the cancer has reached the lymph nodes, it may mean that cancer cells have spread to other parts of the body such as the liver, lungs or brain, giving rise to metastatic melanoma.

[0006] Melanoma is currently diagnosed by assessing risk factors and by performing biopsies. Risk factors for melanoma are a family history of melanoma, the presence of dysplastic nevi, patient history of melanoma, weakened immune system, many ordinary nevi, exposure levels to ultraviolet radiation, exposure to severe sunburns especially as a child or teenager, and fair skin. In a biopsy, a pathologist typically examines the biopsied tissue under a microscope to identify cancer cells. Depending upon the thickness of a tumor, if one exists, a physician may order chest x-ray, blood tests, liver scans, bone scans, and brain scans to determine whether the cancer spread to other tissues. Also, a test that identifies p16 nucleotide sequences is sold.

[0007] Upon a diagnosis of melanoma, the standard treatment is surgery. Side effects of surgery typically are pain and scarring. Surgery is generally not effective, however, in controlling melanoma that is known to have spread to other parts of the body. In such cases, physicians may utilize other methods of treatment, such as chemotherapy, biological therapy, radiation therapy, or a combination of these methods. Chemotherapy agents for treating melanoma include cisplatin, vinblastine, and dacarbazine. Chemotherapy can lead to side effects such as an increased probability of infection, bruising and bleeding, weakness and fatigue, hair loss, poor appetite, nausea and vomiting, and mouth and lip sores. Side effects of radiation therapy include fatigue and hair loss in the treated area. Biological therapies currently utilized for treatment of melanoma include interferon and interleuken-2. Side effects caused by biological therapies include flu-like symptoms, such as chills, fever, muscle aches, weakness, loss of appetite, nausea, vomiting, and diarrhea; bleeding and bruising skin; rashes, and swelling.

[0008] Certain melanoma therapeutics are in clinical trials. For example, canvaxin, which is a whole cell allogenic vaccine developed by irradiating tumor cells from two different patients, is under study. In addition, MAGE-1 and 3 minigenes and peptides and gp100 peptides are being tested. Upcoming studies include testing of agents such as dacarbazine with a bcl-2 antisense oligonucleotide, and paclitaxel in combination with a matrix metalloprotease inhibitor.

Summary

[0009] It has been discovered that polymorphic variations in a gene encoding a protein kinase known as *BRAF* is associated with the occurrence of melanoma. Thus, featured herein are methods for identifying a subject at risk of melanoma and determining risk of melanoma in a subject, which comprise detecting the presence or absence of one or more polymorphic variations associated with melanoma in a nucleic acid sample from a subject. The polymorphism often is detected in or near the *BRAF* nucleotide sequence, which is set forth as SEQ ID NO: 1, or a substantially identical nucleotide sequence thereof. In embodiments, polymorphic variations at positions 146311, 138875, 132526, 128002, 118712, 98846, 98682, 87826, 80400, 76779, 68398 and 64547 of SEQ ID NO: 1 may be detected.

[0010] Also featured herein are nucleic acids that encode a *BRAF* polypeptide, and variants thereof, and include one or more polymorphic variations selected from positions 146311, 138875, 132526, 128002, 118712, 98846, 98682, 87826, 80400, 76779, 68398 and 64547 in SEQ ID NO: 1, and oligonucleotides which hybridize to those nucleic acids. Also provided are polypeptides encoded by nucleic acids having a *BRAF* nucleotide sequence, which include the full-length polypeptide, isoforms and fragments thereof. In addition, provided herein are methods for identifying candidate therapeutic molecules for treating melanoma and related disorders, as well as methods of treating melanoma in a subject by administering a therapeutic molecule.

Brief Description Of The Drawings

[0011] Figures 1A-1AAA depict the *BRAF* nucleotide sequence reported as SEQ ID NO: 1. The following nucleotide representations are used throughout: "A" or "a" is adenosine, adenine, or adenylic acid; "C" or "c" is cytidine, cytosine, or cytidylic acid; "G" or "g" is guanosine, guanine, or guanylic acid; "T" or "t" is thymidine, thymine, or thymidylic acid; and "I" or "i" is inosine, hypoxanthine, or inosinic acid. Exons are indicated in italicized lower case type, introns are depicted in normal text lower case type, and polymorphic sites are depicted in bold upper case type. SNPs are designated by the following convention: "R" represents A or G, "M" represents A or C; "W" represents A or T; "Y" represents C or T; "S" represents C or G; "K" represents G or T; "V" represents A, C or G; "H" represents A, C, or T; "D" represents A, G, or T; "B" represents C, G, or T; and "N" represents A, G, C, or T.

[0012] Figures 2A-2G show polypeptide sequences encoded by the nucleic acid of SEQ ID NO: 1, which correspond to the polypeptides a-g described in Table 1, respectively.

[0013] Figures 3A, 3C, and 3E depict nucleotide sequences of cDNA fragments corresponding to human, rat, and mouse *BRAF*, respectively. Figures 3B, 3D, and 3F depict polypeptide sequences encoded by the cDNA fragments of Figures 3A, 3C, and 3E, respectively.

[0014] Figure 4A-4D illustrate an alignment of the human, rat, and mouse cDNA sequences.

[0015] Figures 5A-5D depict an alignment of the human and mouse cDNA fragments, which shows that there is 64.79% sequence identity between the two sequences.

[0016] Figures 6A-6D are an alignment of the human and rat cDNA fragments, which shows that there is 55.27% sequence identity between the two sequences.

[0017] Figures 7A to 7F depict amino acid sequences corresponding to human, mouse, rat, *Arabidopsis thalina*, *C. elegans*, and *Drosophila BRAF* polypeptides, respectively.

[0018] Figures 8A and 8B are an alignment of the polypeptide sequences of Figures 7A to 7F, which show that the human and mouse sequences have 53% sequence identity; the human and rat sequences have 54% sequence identity; the human and *Arabidopsis* sequences have 22% sequence identity; the human and *C. elegans* sequences have 33% sequence identity; the human and *Drosophila*

sequences have 43% sequence identity; the mouse and rat sequences have 98% sequence identity; the mouse and *Arabidopsis* sequences have 21% sequence identity; the mouse and *C. elegans* sequences have 35% sequence identity; the mouse and *Drosophila* sequences have 53% sequence identity;

[0019] Figure 9 depicts a spacing interval distribution of SNPs in the set of approximately 25,000 SNPs used in the genetic screens described herein.

Detailed Description

[0020] It has been discovered that polymorphic variants in *BRAF* are associated with occurrence of melanoma in subjects. Thus, detecting genetic determinants associated with an increased risk of melanoma occurrence can lead to early identification of melanoma or susceptibility to melanoma and early prescription of preventative measures. Also, associating *BRAF* polymorphic variants with melanoma has provided new targets for screening molecules useful in treatments of melanoma.

Melanoma and Sample Selection

[0021] Melanoma is typically described as a malignant neoplasm derived from cells that are capable of forming melanin. Melanomas arise most commonly in the skin of any part of the body, or in the eye, and rarely, in the mucous membranes of the genitalia, anus, oral cavity, or other sites. Melanoma occurs mostly in adults and may originate de novo or from a pigmented nevus or lentigo maligna. Melanomas frequently metastasize widely to regions such as lymph-nodes, skin, liver, lungs, and brain.

[0022] In the early phases, the cutaneous form is characterized by proliferation of cells at the dermal-epidermal junction that soon invade adjacent tissues. The cells vary in amount and pigmentation of cytoplasm; the nuclei are relatively large and irregular in shape, with prominent acidophilic nucleoli; and mitotic figures tend to be numerous. Other criteria for melanomas are asymmetry, irregular borders, heterogeneous color, large diameter, and a recent change in shape, size or pigmentation. Excised melanoma skin samples are often subjected to the following analyses: diagnosis of the melanocytic nature of the lesion and confirmation of its malignancy; maximum tumor thickness in millimeters (according to Breslow's method); assessment of completeness of excision of invasive and in situ components and microscopic measurements of the shortest extent of clearance; level of invasion (Clark); presence and extent of regression; presence and extent of ulceration; histological type and special variants; pre-existing lesion; mitotic rate; vascular invasion; neurotropism; cell type; tumor lymphocyte infiltration; and growth phase, vertical or radial.

[0023] Based in part upon selection criteria set forth above, individuals having melanoma can be selected for genetic studies. Also, individuals having no history of cancer or melanoma often are selected for genetic studies. Other selection criteria can include: a tissue or fluid sample is derived from an individual characterized as Caucasian; a sample is derived from an individual of German

paternal and maternal descent; and relevant phenotype information is available for the individual. Phenotype information corresponding to each individual can include sex of the individual, number of nevi (*e.g.*, actual number or relative number (*e.g.*, few, moderate, numerous)), hair color (*e.g.*, black, brown, blond, red), diagnosis of melanoma (*e.g.*, tumor thickness, date of primary diagnosis, age of individual as of primary diagnosis, post-operative tumor classification, presence of nodes, occurrence of metastases, subtype, location), country or origin of mother and father, presence of certain conditions for each individual (*e.g.*, coronary heart disease, cardiomyopathy, arteriosclerosis, abnormal blood clotting/thrombosis, emphysema, asthma, diabetes type 1, diabetes type 2, Alzheimer's disease, epilepsy, schizophrenia, manic depression/bipolar disorder, autoimmune disease, thyroid disorder, and hypertension), presence of cancer in the donor individual or blood relative (*e.g.*, melanoma, basaliom/spinaliom/lentigo malignant/mycosis fungoides, breast cancer, colon cancer, rectum cancer, lung cancer, lung cancer, bronchus cancer, prostate cancer, stomach cancer, leukemia, lymphoma, or other cancer in donor, donor parent, donor aunt or uncle, donor offspring or donor grandparent).

[0024] Provided herein is a set of blood samples and a set of corresponding nucleic acid samples isolated from the blood samples, where the blood samples are donated from individuals diagnosed with melanoma. The sample set often includes blood samples or nucleic acid samples from 100 or more, 150 or more, or 200 or more individuals having melanoma, and sometimes from 250 or more, 300 or more, 400 or more, or 500 or more individuals. The individuals can have parents from any place of origin, and in an embodiment, the set of samples are extracted from individuals of German paternal and German maternal ancestry. The samples in each set may be selected based upon five or more criteria and/or phenotypes set forth above.

Polymorphic Variants Associated with Melanoma

[0025] A genetic analysis provided herein linked melanoma with polymorphic variants of a nucleotide sequence located on chromosome seven that encodes a serine/threonine protein kinase polypeptide designated *BRAF*. The *BRAF* gene is located on chromosome 7q34 (assembly 30), and covers approximately 190 kb. It contains at least 19 exons and encodes a full-length transcript of 2510 bp (NM_004333). At least seven variant transcripts have been identified, which are the product of alternative splicing. From these various transcripts, several proteins are translated, including the full-length, 94-95 kD, 783 amino acid product (*see* http address at www.ncbi.nlm.nih.gov/LocusLink/).

[0026] *BRAF* is a member of the *RAF* family, which includes *ARAF* and *CRAF* in humans (Ikawa, *Mol Cell Biol. 8(6)*:2651-4 (1988)). *BRAF* is a serine/threonine protein kinase and participates in the *RAS/RAF/MEK/ERK* MAP kinase signal transduction pathway (Williams & Roberts, *Cancer Metastasis Rev. 13(1)*:105-16 (1994)). This pathway plays a significant role in

modulating cellular responses to extracellular stimuli, particularly in response to growth factors, and the pathway controls cellular events including cell proliferation, cell-cycle arrest, terminal differentiation and apoptosis (Peyssonnaux *et al., Biol Cell. 93(1-2)*:53-62 (2001)). Activation of this pathway via the RAS receptor-ligand results in cytoplasmic *BRAF* protein being localized to the intracellular membrane surface by binding directly to RAS (Jaiswal *et al., Mol Cell Biol. 14(10)*:6944-53 (1994)), which results in *BRAF* phosphorylation. Once phosphorylated, *BRAF* serine/threonine kinase activity is activated and the activated enzyme phosphorylates MEK, which is also referred to as MAPKK. MEK phosphorylation activates its kinase activity, and it in turn phosphorylates ERK, which is also referred to as MAPK. Upon phosphorylation, ERK is translocated into the nucleus, where it phosphorylates transcription factors and thereby stimulates transcription of various genes involved in cell growth, differentiation and apoptosis (Peyssonnaux *et al., Biol Cell. 93(1-2)*:53-62 (2001)). It was also reported that the *BRAF* gene was mutated in individuals having different types of cancers (Davies *et al., Nature 417(6892)*:949-54 (2002)).

[0027] *BRAF* is expressed in numerous tissues, although it is most highly expressed in neural tissue (Barnier *et al., J Biol Chem. 270(40)*:23381-9 (1995)). Of the three *RAF* family members, *CRAF* is the most ubiquitously expressed, but it does not play a major role in MEK activation. *ARAF* is also a poor MEK activator. *BRAF* is the major MEK activator in the *RAF* family, even in cells where its expression level is relatively low (Peyssonnaux *et al., Biol Cell. 93(1-2)*:53-62 (2001)).

[0028] *BRAF* deletion mice were shown to be embryonic lethal, dying at midgestation. This embryonic lethality is marked by a pronounced loss of vascular endothelium integrity and increased apoptosis of the vascular endothelium (Wojnowski *et al., Nat Genet. 16(3)*:293-7 (1997)). It was also reported that *BRAF* interacted with Bcl-2 family members, which are outside of the *RAS/RAF/MEK/ERK* MAP kinase signaling pathway (Wang *et al., Cell 87*: 629-638 (1996)).

[0029] Polymorphic variations at particular polymophic sites in and around *BRAF* were associated with melanoma. As used herein, the term "polymorphic site" refers to a region in a nucleic acid at which two or more alternative nucleotide sequences are observed in a significant number of nucleic acid samples from a population of individuals. A polymorphic site may be a nucleotide sequence of two or more nucleotides, an inserted nucleotide or nucleotide sequence, a deleted nucleotide or nucleotide sequence, or a microsatellite, for example. A polymorphic site that is two or more nucleotides in length may be 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15 or more, 20 or more, 30 or more, 50 or more, 75 or more, 100 or more, 500 or more, or about 1000 nucleotides in length, where all or some of the nucleotide sequences differ within the region. A polymorphic site is often one nucleotide in length, which is referred to herein as a "single nucleotide polymorphism" or a "SNP."

[0030] Where there are two, three, or four alternative nucleotide sequences at a polymorphic site, each nucleotide sequence is referred to as a "polymorphic variant." Where two polymorphic variants exist, for example, the polymorphic variant represented in a minority of samples from a

population is sometimes referred to as a "minor allele" and the polymorphic variant that is more prevalently represented is sometimes referred to as a "major allele." Many organisms possess a copy of each chromosome (*e.g.*, humans), and those individuals who possess two major alleles or two minor alleles are often referred to as being "homozygous" with respect to the polymorphism, and those individuals who possess one major allele and one minor allele are normally referred to as being "heterozygous" with respect to the polymorphism. Individuals who are homozygous with respect to one allele are sometimes predisposed to a different phenotype as compared to individuals who are heterozygous or homozygous with respect to another allele.

[0031] Furthermore, a genotype or polymorphic variant may be expressed in terms of a "haplotype," which as used herein refers to two or more polymorphic variants occurring within genomic DNA in a group of individuals within a population. For example, two SNPs may exist within a gene where each SNP position includes a cytosine variation and an adenine variation. Certain individuals in a population may carry one allele (heterozygous) or two alleles (homozygous) having the gene with a cytosine at each SNP position. As the two cytosines corresponding to each SNP in the gene travel together on one or both alleles in these individuals, the individuals can be characterized as having a cytosine/cytosine haplotype with respect to the two SNPs in the gene.

[0032] As used herein, the term "phenotype" refers to a trait which can be compared between individuals, such as presence or absence of a condition, a visually observable difference in appearance between individuals, metabolic variations, physiological variations, variations in the function of biological molecules, and the like. An example of a phenotype is occurrence of melanoma.

[0033] Researchers sometimes report a polymorphic variant in a database without determining whether the variant is represented in a significant fraction of a population. Because a subset of these reported polymorphic variants are not represented in a statistically significant portion of the population, some of them are sequencing errors and/or not biologically relevant. Thus, it is often not known whether a reported polymorphic variant is statistically significant or biologically relevant until the presence of the variant is detected in a population of individuals and the frequency of the variant is determined. Methods for detecting a polymorphic variant in a population are described herein, specifically in Example 2. A polymorphic variant is statistically significant and often biologically relevant if it is represented in 5% or more of a population, sometimes 10% or more, 15% or more, or 20% or more of a population, and often 25% or more, 30% or more, 35% or more, 40% or more, 45% or more, or 50% or more of a population.

[0034] A polymorphic variant may be detected on either or both strands of a double-stranded nucleic acid. Also, a polymorphic variant may be located within an intron or exon of a gene or within a portion of a regulatory region such as a promoter, a 5′ untranslated region (UTR), a 3′ UTR, and in DNA (*e.g.*, genomic DNA (gDNA) and complementary DNA (cDNA)), RNA (*e.g.*, mRNA, tRNA,

and rRNA), or a polypeptide. Polymorphic variations may or may not result in detectable differences in gene expression, polypeptide structure, or polypeptide function.

[0035] For duplex DNA, a polymorphic variation may be reported from one strand or its complementary strand. For example, a thymine at position 138875 in SEQ ID NO: 1 can be reported as an adenine from the complementary strand. Also, while polymorphic variations at all positions within a haplotype often are reported from the same strand orientation, polymorphic variations at certain positions within a haplotype sometimes are reported from one strand orientation while others are reported from the other. The latter sometimes occurs even though it is understood by the person of ordinary skill in the art that polymorphic variants in a haplotype occur within one strand in a nucleic acid. Where a haplotype is reported from mixed strand orientations, a person of ordinary skill in the art can determine the orientation of each polymorphic variation in the haplotype by analyzing the orientation of each extension olig890nucleotide (*e.g.*, Table 6) utilized to identify each polymorphic variation. For example, a person of ordinary skill in the art would understand that the H4 haplotype CTTG reported in Table 12 of Example 2 (corresponding to positions 146311, 138875, 76779, and 68398, respectively, in SEQ ID NO: 1) could be reported as CATG in view of the orientation of the extension oligonucleotides set forth in Table 6, since the extension oligonucleotide used to identify the polymorphism at position 138875 is in a reverse orientation as compared to the extension oligonucleotides used to identify polymorphisms at positions 146311, 76779, and 68398.

[0036] In the genetic analysis that associated polymorphic variations in *BRAF* with melanoma, samples from individuals having melanoma and individuals not having cancer were allelotyped and genotyped. The term "allelotype" as used herein refers to a process for determining the allele frequency for a polymorphic variant in pooled DNA samples from cases and controls. By pooling DNA from each group, an allele frequency for each SNP in each group is calculated. These allele frequencies are then compared to one another. Particular SNPs are considered as being associated with a particular disease when allele frequency differences calculated between case and control pools are statistically significant. The term "genotyped" as used herein refers to a process for determining a genotype of one or more individuals, where a "genotype" is a representation of one or more polymorphic variants in a population. It was determined that SNPs existed in the *BRAF* nucleic acid at positions 146311, 138875, 132526, 128002, 118712, 98846, 98682, 87826, 80400, 76779, 68398 and 64547 of the individuals tested in the genetic analysis. It was also determined that the occurrence or predisposition of melanoma was associated with males and females having the haplotype CTTG (corresponding to positions 146311, 138875, 76779, and 68398, respectively, in SEQ ID NO: 1), males having the haplotype ATGA (same nomenclature as for the CTTG haplotype), and males having an adenine at position 146311 of SEQ ID NO: 1 (reported in the reverse orientation of the *BRAF* gene (i.e., from the strand set forth in Figure 1)). Also, predisposition to melanoma was associated in males and females having the haplotypes GGTTCGCATACT and GGTTCGTATATC,

in females having the haplotype GATTCGCATACC, and in males having the haplotype TACCGATCCCTT (each twelve-position haplotype corresponds to positions 146311, 138875, 132526, 128002, 118712, 98846, 98682, 87826, 80400, 76779, 68398 and 64547, respectively, of SEQ ID NO: 1, and is reported in the forward orientation of the *BRAF* gene (complementary to the sequence in Figure 1)).

Additional Polymorphic Variants Associated with Melanoma

[0037] Also provided is a method for identifying polymorphic variants proximal to an incident, founder polymorphic variant associated with melanoma. Thus, featured herein are methods for identifying a polymorphic variation associated with melanoma that is proximal to an incident polymorphic variation associated with melanoma, which comprises identifying a polymorphic variant proximal to the incident polymorphic variant associated with melanoma, where the incident polymorphic variant is in a nucleotide sequence set forth in SEQ ID NO:1. The nucleotide sequence often comprises a polynucleotide sequence selected from the group consisting of (a) a polynucleotide sequence set forth in SEQ ID NO:1; (b) a polynucleotide sequence that encodes a polypeptide having an amino acid sequence encoded by a nucleotide sequence set forth in SEQ ID NO:1; and (c) a polynucleotide sequence that encodes a polypeptide having an amino acid sequence that is 90% or more identical to an amino acid sequence encoded by a nucleotide sequence set forth in SEQ ID NO:1 or a polynucleotide sequence 90% or more identical to the polynucleotide sequence set forth in SEQ ID NO:1. The presence or absence of an association of the proximal polymorphic variant with NIDDM then is determined using a known association method, such as a method described in the Examples hereafter. In an embodiment, the incident polymorphic variant is described in SEQ ID NO:1 or Table 4. In another embodiment, the proximal polymorphic variant identified sometimes is a publicly disclosed polymorphic variant, which for example, sometimes is published in a publicly available database. In other embodiments, the polymorphic variant identified is not publicly disclosed and is discovered using a known method, including, but not limited to, sequencing a region surrounding the incident polymorphic variant in a group of nucleic samples. Thus, multiple polymorphic variants proximal to an incident polymorphic variant are associated with melanoma using this method.

[0038] The proximal polymorphic variant often is identified in a region surrounding the incident polymorphic variant. In certain embodiments, this surrounding region is about 50 kb flanking the first polymorphic variant (*e.g.* about 50 kb 5' of the first polymorphic variant and about 50 kb 3' of the first polymorphic variant), and the region sometimes is composed of shorter flanking sequences, such as flanking sequences of about 40 kb, about 30 kb, about 25 kb, about 20 kb, about 15 kb, about 10 kb, about 7 kb, about 5 kb, or about 2 kb 5' and 3' of the incident polymorphic variant. In other embodiments, the region is composed of longer flanking sequences, such as flanking

sequences of about 55 kb, about 60 kb, about 65 kb, about 70 kb, about 75 kb, about 80 kb, about 85 kb, about 90 kb, about 95 kb, or about 100 kb 5' and 3' of the incident polymorphic variant.

[0039] In certain embodiments, polymorphic variants associated with melanoma are identified iteratively. For example, a first proximal polymorphic variant is associated with melanoma using the methods described above and then another polymorphic variant proximal to the first proximal polymorphic variant is identified (*e.g.*, publicly disclosed or discovered) and the presence or absence of an association of one or more other polymorphic variants proximal to the first proximal polymorphic variant with melanoma is determined.

[0040] The methods described herein are useful for identifying or discovering additional polymorphic variants that may be used to further characterize a gene, region or loci associated with a condition, a disease (*e.g.*, melanoma), or a disorder. For example, allelotyping or genotyping data from the additional polymorphic variants may be used to identify a functional mutation or a region of linkage disequilibrium. In certain embodiments, polymorphic variants identified or discovered within a region comprising the first polymorphic variant associated with melanoma are genotyped using the genetic methods and sample selection techniques described herein, and it can be determined whether those polymorphic variants are in linkage disequilibrium with the first polymorphic variant. The size of the region in linkage disequilibrium with the first polymorphic variant also can be assessed using these genotyping methods. Thus, provided herein are methods for determining whether a polymorphic variant is in linkage disequilibrium with a first polymorphic variant associated with melanoma, and such information can be used in prognosis methods described herein.

Isolated *BRAF* Nucleic Acids and Variants Thereof

[0041] Featured herein are isolated *BRAF* nucleic acids, which include the nucleic acid having the nucleotide sequence of SEQ ID NO: 1, *BRAF* nucleic acid variants, and substantially identical nucleic acids to the foregoing. Nucleotide sequences of the *BRAF* nucleic acids are sometimes referred to herein as "*BRAF* nucleotide sequences." A "*BRAF* nucleic acid variant" refers to one allele that may have different polymorphic variations as compared to another allele in another subject or the same subject. A polymorphic variation in the *BRAF* nucleic acid variant may be represented on one or both strands in a double-stranded nucleic acid or on one chromosomal complement (heterozygous) or both chromosomal complements (homozygous). A *BRAF* nucleic acid may comprise one or more of the following polymorphic variations: an adenine at position 146311 of SEQ ID NO: 1 or a guanine at the same position in a complementary nucleic acid; the haplotype CTTG corresponding to positions 146311, 138875, 76779, and 68398, respectively, in SEQ ID NO: 1 or the haplotype GAAC in a complementary nucleic acid; the haplotype ATGA corresponding to positions 146311, 138875, 76779, and 68398, respectively, in SEQ ID NO: 1 or the haplotype TACT in a complementary nucleic acid; or the haplotype GATTCGCATACC corresponding to positions 146311, 138875, 132526,

128002, 118712, 98846, 98682, 87826, 80400, 76779, 68398 and 64547, respectively, in SEQ ID NO: 1 or the haplotype CTAAGCGTATGG in a complementary nucleic acid.

[0042] As used herein, the term "nucleic acid" includes DNA molecules (*e.g.*, a complementary DNA (cDNA) and genomic DNA (gDNA)) and RNA molecules (*e.g.*, mRNA, rRNA, siRNA and tRNA) and analogs of DNA or RNA, for example, by use of nucleotide analogs. The nucleic acid molecule can be single-stranded and it is often double-stranded. The term "isolated or purified nucleic acid" refers to nucleic acids that are separated from other nucleic acids present in the natural source of the nucleic acid. For example, with regard to genomic DNA, the term "isolated" includes nucleic acids which are separated from the chromosome with which the genomic DNA is naturally associated. An "isolated" nucleic acid is often free of sequences which naturally flank the nucleic acid (*i.e.*, sequences located at the 5' and/or 3' ends of the nucleic acid) in the genomic DNA of the organism from which the nucleic acid is derived. For example, in various embodiments, the isolated nucleic acid molecule can contain less than about 5 kb, 4 kb, 3 kb, 2 kb, 1 kb, 0.5 kb or 0.1 kb of 5' and/or 3' nucleotide sequences which flank the nucleic acid molecule in genomic DNA of the cell from which the nucleic acid is derived. Moreover, an "isolated" nucleic acid molecule, such as a cDNA molecule, can be substantially free of other cellular material, or culture medium when produced by recombinant techniques, or substantially free of chemical precursors or other chemicals when chemically synthesized. As used herein, the term "*BRAF* gene" refers to a nucleotide sequence that encodes a *BRAF* polypeptide.

[0043] Also included herein are nucleic acid fragments. These fragments are typically a nucleotide sequence identical to a nucleotide sequence in SEQ ID NO: 1, a nucleotide sequence substantially identical to a nucleotide sequence in SEQ ID NO: 1, or a nucleotide sequence that is complementary to the foregoing. The nucleic acid fragment may be identical, substantially identical or homologous to a nucleotide sequence in an exon or an intron in SEQ ID NO: 1 and may encode a full-length or mature polypeptide, or may encode a domain or part of a domain of a *BRAF* polypeptide. Sometimes, the fragment will comprises one or more of the polymorphic variations described herein as being associated with melanoma. The nucleic acid fragment is often 50, 100, or 200 or fewer base pairs in length, and is sometimes about 300, 400, 500, 600, 700, 800, 900, 1000, 1100, 1200, 1300, or 1400 base pairs in length. A nucleic acid fragment that is complementary to a nucleotide sequence identical or substantially identical to the nucleotide sequence of SEQ ID NO: 1 and hybridizes to such a nucleotide sequence under stringent conditions is often referred to as a "probe." Nucleic acid fragments often include one or more polymorphic sites, or sometimes have an end that is adjacent to a polymorphic site as described hereafter.

[0044] An example of a nucleic acid fragment is an oligonucleotide. As used herein, the term "oligonucleotide" refers to a nucleic acid comprising about 8 to about 50 covalently linked nucleotides, often comprising from about 8 to about 35 nucleotides, and more often from about 10 to

about 25 nucleotides. The backbone and nucleotides within an oligonucleotide may be the same as those of naturally occurring nucleic acids, or analogs or derivatives of naturally occurring nucleic acids, provided that oligonucleotides having such analogs or derivatives retain the ability to hybridize specifically to a nucleic acid comprising a targeted polymorphism. Oligonucleotides described herein may be used as hybridization probes or as components of prognostic or diagnostic assays, for example, as described herein.

[0045] Oligonucleotides are typically synthesized using standard methods and equipment, such as the ABI™3900 High Throughput DNA Synthesizer and the EXPEDITE™ 8909 Nucleic Acid Synthesizer, both of which are available from Applied Biosystems (Foster City, CA). Analogs and derivatives are exemplified in U.S. Pat. Nos. 4,469,863; 5,536,821; 5,541,306; 5,637,683; 5,637,684; 5,700,922; 5,717,083; 5,719,262; 5,739,308; 5,773,601; 5,886,165; 5,929,226; 5,977,296; 6,140,482; WO 00/56746; WO 01/14398, and related publications. Methods for synthesizing oligonucleotides comprising such analogs or derivatives are disclosed, for example, in the patent publications cited above and in U.S. Pat. Nos. 5,614,622; 5,739,314; 5,955,599; 5,962,674; 6,117,992; in WO 00/75372; and in related publications.

[0046] Oligonucleotides may also be linked to a second moiety. The second moiety may be an additional nucleotide sequence such as a tail sequence (*e.g.*, a polyadenosine tail), an adapter sequence (*e.g.*, phage M13 universal tail sequence), and others. Alternatively, the second moiety may be a non-nucleotide moiety such as a moiety which facilitates linkage to a solid support or a label to facilitate detection of the oligonucleotide. Such labels include, without limitation, a radioactive label, a fluorescent label, a chemiluminescent label, a paramagnetic label, and the like. The second moiety may be attached to any position of the oligonucleotide, provided the oligonucleotide can hybridize to the nucleic acid comprising the polymorphism.

Uses for Nucleic Acid Sequence

[0047] Nucleic acid coding sequences depicted in SEQ ID NO: 1 and Figures 3A, 3C and 3E may be used for diagnostic purposes for detection and control of polypeptide expression. Also, included herein are oligonucleotide sequences such as antisense RNA, small-interfering RNA (siRNA) and DNA molecules and ribozymes that function to inhibit translation of a polypeptide. Antisense techniques and RNA interference techniques are known in the art and are described herein.

[0048] Ribozymes are enzymatic RNA molecules capable of catalyzing the specific cleavage of RNA. The mechanism of ribozyme action involves sequence specific hybridization of the ribozyme molecule to complementary target RNA, followed by endonucleolytic cleavage. For example, hammerhead motif ribozyme molecules may be engineered that specifically and efficiently catalyze endonucleolytic cleavage of RNA sequences corresponding to or complementary to the nucleotide sequences set forth in Figures 1A and 1B. Specific ribozyme cleavage sites within any potential RNA

target are initially identified by scanning the target molecule for ribozyme cleavage sites which include the following sequences, GUA, GUU and GUC. Once identified, short RNA sequences of between fifteen (15) and twenty (20) ribonucleotides corresponding to the region of the target gene containing the cleavage site may be evaluated for predicted structural features such as secondary structure that may render the oligonucleotide sequence unsuitable. The suitability of candidate targets may also be evaluated by testing their accessibility to hybridization with complementary oligonucleotides, using ribonuclease protection assays.

[0049] Antisense RNA and DNA molecules, siRNA and ribozymes may be prepared by any method known in the art for the synthesis of RNA molecules. These include techniques for chemically synthesizing oligodeoxyribonucleotides well known in the art such as solid phase phosphoramidite chemical synthesis. Alternatively, RNA molecules may be generated by *in vitro* and *in vivo* transcription of DNA sequences encoding the antisense RNA molecule. Such DNA sequences may be incorporated into a wide variety of vectors which incorporate suitable RNA polymerase promoters such as the T7 or SP6 polymerase promoters. Alternatively, antisense cDNA constructs that synthesize antisense RNA constitutively or inducibly, depending on the promoter used, can be introduced stably into cell lines.

[0050] DNA encoding a polypeptide also may have a number of uses for the diagnosis of diseases, including melanoma, resulting from aberrant expression of a target gene described herein. For example, the nucleic acid sequence may be used in hybridization assays of biopsies or autopsies to diagnose abnormalities of expression or function (*e.g.*, Southern or Northern blot analysis, in situ hybridization assays).

[0051] In addition, the expression of a polypeptide during embryonic development may also be determined using nucleic acid encoding the polypeptide. As addressed, *infra*, production of functionally impaired polypeptide is the cause of various disease states, melanoma. *In situ* hybridizations using polypeptide as a probe may be employed to predict problems related to melanoma. Further, as indicated, *infra*, administration of human active polypeptide, recombinantly produced as described herein, may be used to treat disease states related to functionally impaired polypeptide. Alternatively, gene therapy approaches may be employed to remedy deficiencies of functional polypeptide or to replace or compete with dysfunctional polypeptide.

Expression Vectors, Host Cells, and Genetically Engineered Cells

[0052] Provided herein are nucleic acid vectors, often expression vectors, which contain a *BRAF* nucleic acid. As used herein, the term "vector" refers to a nucleic acid molecule capable of transporting another nucleic acid to which it has been linked and can include a plasmid, cosmid, or viral vector. The vector can be capable of autonomous replication or it can integrate into a host DNA.

Viral vectors may include replication defective retroviruses, adenoviruses and adeno-associated viruses for example.

[0053] A vector can include a *BRAF* nucleic acid in a form suitable for expression of the nucleic acid in a host cell. The recombinant expression vector typically includes one or more regulatory sequences operatively linked to the nucleic acid sequence to be expressed. The term "regulatory sequence" includes promoters, enhancers and other expression control elements (*e.g.*, polyadenylation signals). Regulatory sequences include those that direct constitutive expression of a nucleotide sequence, as well as tissue-specific regulatory and/or inducible sequences. The design of the expression vector can depend on such factors as the choice of the host cell to be transformed, the level of expression of polypeptide desired, and the like. Expression vectors can be introduced into host cells to produce *BRAF* polypeptides, including fusion polypeptides, encoded by *BRAF* nucleic acids.

[0054] Recombinant expression vectors can be designed for expression of *BRAF* polypeptides in prokaryotic or eukaryotic cells. For example, *BRAF* polypeptides can be expressed in *E. coli*, insect cells (*e.g.*, using baculovirus expression vectors), yeast cells, or mammalian cells. Suitable host cells are discussed further in Goeddel, *Gene Expression Technology: Methods in Enzymology 185*, Academic Press, San Diego, CA (1990). Alternatively, the recombinant expression vector can be transcribed and translated *in vitro*, for example using T7 promoter regulatory sequences and T7 polymerase.

[0055] Expression of polypeptides in prokaryotes is most often carried out in *E. coli* with vectors containing constitutive or inducible promoters directing the expression of either fusion or non-fusion polypeptides. Fusion vectors add a number of amino acids to a polypeptide encoded therein, usually to the amino terminus of the recombinant polypeptide. Such fusion vectors typically serve three purposes: 1) to increase expression of recombinant polypeptide; 2) to increase the solubility of the recombinant polypeptide; and 3) to aid in the purification of the recombinant polypeptide by acting as a ligand in affinity purification. Often, a proteolytic cleavage site is introduced at the junction of the fusion moiety and the recombinant polypeptide to enable separation of the recombinant polypeptide from the fusion moiety subsequent to purification of the fusion polypeptide. Such enzymes, and their cognate recognition sequences, include Factor Xa, thrombin and enterokinase. Typical fusion expression vectors include pGEX (Pharmacia Biotech Inc; Smith & Johnson, *Gene 67:* 31-40 (1988)), pMAL (New England Biolabs, Beverly, MA) and pRIT5 (Pharmacia, Piscataway, NJ) which fuse glutathione S-transferase (GST), maltose E binding polypeptide, or polypeptide A, respectively, to the target recombinant polypeptide.

[0056] Purified fusion polypeptides can be used in screening assays and to generate antibodies specific for *BRAF* polypeptides. In a therapeutic embodiment, fusion polypeptide expressed in a retroviral expression vector is used to infect bone marrow cells that are subsequently transplanted into

irradiated recipients. The pathology of the subject recipient is then examined after sufficient time has passed (*e.g.*, six (6) weeks).

[0057] Expressing the polypeptide in host bacteria with an impaired capacity to proteolytically cleave the recombinant polypeptide is often used to maximize recombinant polypeptide expression (Gottesman, S., *Gene Expression Technology: Methods in Enzymology, Academic Press, San Diego, California 185:* 119-128 (1990)). Another strategy is to alter the nucleotide sequence of the nucleic acid to be inserted into an expression vector so that the individual codons for each amino acid are those preferentially utilized in *E. coli* (Wada *et al., Nucleic Acids Res. 20:* 2111-2118 (1992)). Such alteration of nucleotide sequences can be carried out by standard DNA synthesis techniques.

[0058] When used in mammalian cells, the expression vector's control functions are often provided by viral regulatory elements. For example, commonly used promoters are derived from polyoma, Adenovirus 2, cytomegalovirus and Simian Virus 40. Recombinant mammalian expression vectors are often capable of directing expression of the nucleic acid in a particular cell type (*e.g.*, tissue-specific regulatory elements are used to express the nucleic acid). Non-limiting examples of suitable tissue-specific promoters include an albumin promoter (liver-specific; Pinkert *et al., Genes Dev. 1:* 268-277 (1987)), lymphoid-specific promoters (Calame & Eaton, *Adv. Immunol. 43:* 235-275 (1988)), promoters of T cell receptors (Winoto & Baltimore, *EMBO J. 8:* 729-733 (1989)) promoters of immunoglobulins (Banerji *et al., Cell 33:* 729-740 (1983); Queen & Baltimore, *Cell 33:* 741-748 (1983)), neuron-specific promoters (*e.g.*, the neurofilament promoter; Byrne & Ruddle, *Proc. Natl. Acad. Sci. USA 86:* 5473-5477 (1989)), pancreas-specific promoters (Edlund *et al., Science 230:* 912-916 (1985)), and mammary gland-specific promoters (*e.g.*, milk whey promoter; U.S. Patent No. 4,873,316 and European Application Publication No. 264,166). Developmentally-regulated promoters are sometimes utilized, for example, the murine hox promoters (Kessel & Gruss, *Science 249:* 374-379 (1990)) and the α-fetopolypeptide promoter (Campes & Tilghman, *Genes Dev. 3:* 537-546 (1989)).

[0059] A *BRAF* nucleic acid may also be cloned into an expression vector in an antisense orientation. Regulatory sequences (*e.g.*, viral promoters and/or enhancers) operatively linked to a *BRAF* nucleic acid cloned in the antisense orientation can be chosen for directing constitutive, tissue specific or cell type specific expression of antisense RNA in a variety of cell types. Antisense expression vectors can be in the form of a recombinant plasmid, phagemid or attenuated virus. For a discussion of the regulation of gene expression using antisense genes see Weintraub *et al.*, Antisense RNA as a molecular tool for genetic analysis, *Reviews - Trends in Genetics*, Vol. 1(1) (1986).

[0060] Also provided herein are host cells that include a *BRAF* nucleic acid within a recombinant expression vector or *BRAF* nucleic acid sequence fragments which allow it to homologously recombine into a specific site of the host cell genome. The terms "host cell" and "recombinant host cell" are used interchangeably herein. Such terms refer not only to the particular

subject cell but rather also to the progeny or potential progeny of such a cell. Because certain modifications may occur in succeeding generations due to either mutation or environmental influences, such progeny may not, in fact, be identical to the parent cell, but are still included within the scope of the term as used herein. A host cell can be any prokaryotic or eukaryotic cell. For example, a *BRAF* polypeptide can be expressed in bacterial cells such as *E. coli*, insect cells, yeast or mammalian cells (such as Chinese hamster ovary cells (CHO) or COS cells). Other suitable host cells are known to those skilled in the art.

[0061] Vectors can be introduced into host cells via conventional transformation or transfection techniques. As used herein, the terms "transformation" and "transfection" are intended to refer to a variety of art-recognized techniques for introducing foreign nucleic acid (*e.g.*, DNA) into a host cell, including calcium phosphate or calcium chloride co-precipitation, transduction/infection, DEAE-dextran-mediated transfection, lipofection, or electroporation.

[0062] A host cell provided herein can be used to produce (*i.e.*, express) a *BRAF* polypeptide. Accordingly, further provided are methods for producing a *BRAF* polypeptide using the host cells described herein. In one embodiment, the method includes culturing host cells into which a recombinant expression vector encoding a *BRAF* polypeptide has been introduced in a suitable medium such that a *BRAF* polypeptide is produced. In another embodiment, the method further includes isolating a *BRAF* polypeptide from the medium or the host cell.

[0063] Also provided are cells or purified preparations of cells which include a *BRAF* transgene, or which otherwise misexpress *BRAF* polypeptide. Cell preparations can consist of human or non-human cells, *e.g.*, rodent cells, *e.g.*, mouse or rat cells, rabbit cells, or pig cells. In embodiments, the cell or cells include a *BRAF* transgene (*e.g.*, a heterologous form of a *BRAF* such as a human gene expressed in non-human cells). The *BRAF* transgene can be misexpressed, *e.g.*, overexpressed or underexpressed. In other embodiments, the cell or cells include a gene which misexpress an endogenous *BRAF* polypeptide (*e.g.*, expression of a gene is disrupted, also known as a knockout). Such cells can serve as a model for studying disorders which are related to mutated or mis-expressed *BRAF* alleles or for use in drug screening. Also provided are human cells (*e.g.*, a hematopoietic stem cells) transformed with a *BRAF* nucleic acid.

[0064] Also provided are cells or a purified preparation thereof (*e.g.*, human cells) in which an endogenous *BRAF* nucleic acid is under the control of a regulatory sequence that does not normally control the expression of the endogenous *BRAF* gene. The expression characteristics of an endogenous gene within a cell (*e.g.*, a cell line or microorganism) can be modified by inserting a heterologous DNA regulatory element into the genome of the cell such that the inserted regulatory element is operably linked to the endogenous *BRAF* gene. For example, an endogenous *BRAF* gene (*e.g.*, a gene which is "transcriptionally silent," not normally expressed, or expressed only at very low levels) may be activated by inserting a regulatory element which is capable of promoting the

expression of a normally expressed gene product in that cell. Techniques such as targeted homologous recombinations, can be used to insert the heterologous DNA as described in, *e.g.*, Chappel, US 5,272,071; WO 91/06667, published on May 16, 1991.

Transgenic Animals

[0065] Non-human transgenic animals that express a heterologous *BRAF* polypeptide (*e.g.*, expressed from a *BRAF* nucleic acid isolated from another organism) can be generated. Such animals are useful for studying the function and/or activity of a *BRAF* polypeptide and for identifying and/or evaluating modulators of *BRAF* nucleic acid and *BRAF* polypeptide activity. As used herein, a "transgenic animal" is a non-human animal such as a mammal (*e.g.*, a non-human primate such as chimpanzee, baboon, or macaque; an ungulate such as an equine, bovine, or caprine; or a rodent such as a rat, a mouse, or an Israeli sand rat), a bird (*e.g.*, a chicken or a turkey), an amphibian (*e.g.*, a frog, salamander, or newt), or an insect (*e.g.*, *Drosophila melanogaster*), in which one or more of the cells of the animal includes a *BRAF* transgene. A transgene is exogenous DNA or a rearrangement (*e.g.*, a deletion of endogenous chromosomal DNA) that is often integrated into or occurs in the genome of cells in a transgenic animal. A transgene can direct expression of an encoded gene product in one or more cell types or tissues of the transgenic animal, and other transgenes can reduce expression (*e.g.*, a knockout). Thus, a transgenic animal can be one in which an endogenous *BRAF* gene has been altered by homologous recombination between the endogenous gene and an exogenous DNA molecule introduced into a cell of the animal (*e.g.*, an embryonic cell of the animal) prior to development of the animal.

[0066] Intronic sequences and polyadenylation signals can also be included in the transgene to increase expression efficiency of the transgene. One or more tissue-specific regulatory sequences can be operably linked to a *BRAF* transgene to direct expression of a *BRAF* polypeptide to particular cells. A transgenic founder animal can be identified based upon the presence of a *BRAF* transgene in its genome and/or expression of *BRAF* mRNA in tissues or cells of the animals. A transgenic founder animal can then be used to breed additional animals carrying the transgene. Moreover, transgenic animals carrying a transgene encoding a *BRAF* polypeptide can further be bred to other transgenic animals carrying other transgenes.

[0067] *BRAF* polypeptides can be expressed in transgenic animals or plants by introducing, for example, a nucleic acid encoding the polypeptide into the genome of an animal. In embodiments the nucleic acid is placed under the control of a tissue specific promoter, *e.g.*, a milk or egg specific promoter, and recovered from the milk or eggs produced by the animal. Also included is a population of cells from a transgenic animal.

BRAF Polypeptides

[0068] Also featured herein are isolated *BRAF* polypeptides, which include polypeptides having amino acid sequences set forth in Figures 2A-2G (SEQ ID NO:), and substantially identical polypeptides thereof. Isolated BRAF polypeptides featured herein include both the full-length polypeptide and the mature polypeptide (*i.e.*, the polypeptide minus the signal sequence or propeptide domain). Characteristics of the polypeptides set forth in Figures 2A-2G are depicted in Table 1. A *BRAF* polypeptide is a polypeptide encoded by a *BRAF* nucleic acid, where one nucleic acid can encode one or more different polypeptides. An "isolated" or "purified" polypeptide or protein is substantially free of cellular material or other contaminating proteins from the cell or tissue source from which the protein is derived, or substantially free from chemical precursors or other chemicals when chemically synthesized. In one embodiment, the language "substantially free" means preparation of a *BRAF* polypeptide or *BRAF* polypeptide variant having less than about 30%, 20%, 10% and more preferably 5% (by dry weight), of non-*BRAF* polypeptide (also referred to herein as a "contaminating protein"), or of chemical precursors or non-*BRAF* chemicals. When the *BRAF* polypeptide or a biologically active portion thereof is recombinantly produced, it is also preferably substantially free of culture medium, specifically, where culture medium represents less than about 20%, sometimes less than about 10%, and often less than about 5% of the volume of the polypeptide preparation. Isolated or purified *BRAF* polypeptide preparations are sometimes 0.01 milligrams or more or 0.1 milligrams or more, and often 1.0 milligrams or more and 10 milligrams or more in dry weight.

TABLE 1

Variant	Transcript length	Exons used	Protein length (aa)	mRNA coordinates
a	2934	19	783	1-2352
b	2657	19	685	1-2058
c	514	3	100	2-304
d	1067	4	220	1-663
e	1172	3	182	3-551
f	2265	1	79	1390-1629
g	1887	2	74	2-226

[0069] Further included herein are *BRAF* polypeptide fragments. The polypeptide fragment may be a domain or part of a domain of a *BRAF* polypeptide. The polypeptide fragment may have increased, decreased or unexpected biological activity. *BRAF* domains include, but are not limited to, Raf-like Ras-binding domain at about amino acids positions 155 to 227, phorbol esters/diacylglycerol binding domain at about amino acids 235 to 280, and protein kinase domain at about amino acids 457 to 714. The polypeptide fragment is often 50 or fewer, 100 or fewer, or 200 or fewer amino acids in length, and is sometimes 300, 400, 500, 600, or 700, or fewer amino acids in length.

[0070] Substantially identical polypeptides may depart from the amino acid sequences set forth in Figures 2A-2G in different manners. For example, conservative amino acid modifications may be introduced at one or more positions in the amino acid sequences of Figures 2A-2G. A "conservative amino acid substitution" is one in which the amino acid is replaced by another amino acid having a similar structure and/or chemical function. Families of amino acid residues having similar structures and functions are well known. These families include amino acids with basic side chains (*e.g.*, lysine, arginine, histidine), acidic side chains (*e.g.*, aspartic acid, glutamic acid), uncharged polar side chains (*e.g.*, glycine, asparagine, glutamine, serine, threonine, tyrosine, cysteine), nonpolar side chains (*e.g.*, alanine, valine, leucine, isoleucine, proline, phenylalanine, methionine, tryptophan), beta-branched side chains (*e.g.*, threonine, valine, isoleucine) and aromatic side chains (*e.g.*, tyrosine, phenylalanine, tryptophan, histidine). Also, essential and non-essential amino acids may be replaced. A "non-essential" amino acid is one that can be altered without abolishing or substantially altering the biological function of a *BRAF* polypeptide, whereas altering an "essential" amino acid abolishes or substantially alters the biological function of a *BRAF* polypeptide. Amino acids that are conserved among *BRAF* polypeptides are typically essential amino acids.

[0071] Also, *BRAF* polypeptides and polypeptide variants may exist as chimeric or fusion polypeptides. As used herein, a *BRAF* "chimeric polypeptide" or "fusion polypeptide" includes a *BRAF* polypeptide linked to a non-*BRAF* polypeptide. A "non-*BRAF* polypeptide" refers to a polypeptide having an amino acid sequence corresponding to a polypeptide which is not substantially identical to the *BRAF* polypeptide, which includes, for example, a polypeptide that is different from the *BRAF* polypeptide and derived from the same or a different organism. The *BRAF* polypeptide in the fusion polypeptide can correspond to an entire or nearly entire *BRAF* polypeptide or a fragment thereof. The non-*BRAF* polypeptide can be fused to the N-terminus or C-terminus of the *BRAF* polypeptide.

[0072] Fusion polypeptides can include a moiety having high affinity for a ligand. For example, the fusion polypeptide can be a GST-*BRAF* fusion polypeptide in which the *BRAF* sequences are fused to the C-terminus of the GST sequences, or a polyhistidine-*BRAF* fusion polypeptide in which the *BRAF* polypeptide is fused at the N- or C-terminus to a string of histidine residues. Such fusion polypeptides can facilitate purification of recombinant *BRAF*. Expression vectors are commercially available that already encode a fusion moiety (*e.g.*, a GST polypeptide), and a *BRAF* nucleic acid can be cloned into an expression vector such that the fusion moiety is linked in-frame to the *BRAF* polypeptide. Further, the fusion polypeptide can be a *BRAF* polypeptide containing a heterologous signal sequence at its N-terminus. In certain host cells (*e.g.*, mammalian host cells), expression, secretion, cellular internalization, and cellular localization of a *BRAF* polypeptide can be increased through use of a heterologous signal sequence. Fusion polypeptides can

also include all or a part of a serum polypeptide (*e.g.*, an IgG constant region or human serum albumin).

[0073] *BRAF* polypeptides or fragments thereof can be incorporated into pharmaceutical compositions and administered to a subject *in vivo*. Administration of these *BRAF* polypeptides can be used to affect the bioavailability of a *BRAF* substrate and may effectively increase *BRAF* biological activity in a cell. *BRAF* fusion polypeptides may be useful therapeutically for the treatment of disorders caused by, for example, (i) aberrant modification or mutation of a gene encoding a *BRAF* polypeptide; (ii) mis-regulation of the *BRAF* gene; and (iii) aberrant post-translational modification of a *BRAF* polypeptide. Also, *BRAF* polypeptides can be used as immunogens to produce anti-*BRAF* antibodies in a subject, to purify *BRAF* ligands or binding partners, and in screening assays to identify molecules which inhibit or enhance the interaction of *BRAF* with a *BRAF* substrate.

[0074] In addition, polypeptides can be chemically synthesized using techniques known in the art (See, *e.g.*, Creighton, 1983 Proteins. New York, N.Y.: W. H. Freeman and Company; and Hunkapiller et al., (1984) *Nature* July 12 -18;310(5973):105-11). For example, a relative short fragment can be synthesized by use of a peptide synthesizer. Furthermore, if desired, nonclassical amino acids or chemical amino acid analogs can be introduced as a substitution or addition into the fragment sequence. Non-classical amino acids include, but are not limited to, to the D-isomers of the common amino acids, 2,4-diaminobutyric acid, a-amino isobutyric acid, 4-aminobutyric acid, Abu, 2-amino butyric acid, g-Abu, e-Ahx, 6-amino hexanoic acid, Aib, 2-amino isobutyric acid, 3-amino propionic acid, ornithine, norleucine, norvaline, hydroxyproline, sarcosine, citrulline, homocitrulline, cysteic acid, t-butylglycine, t-butylalanine, phenylglycine, cyclohexylalanine, b-alanine, fluoroamino acids, designer amino acids such as b-methyl amino acids, Ca-methyl amino acids, Na-methyl amino acids, and amino acid analogs in general. Furthermore, the amino acid can be D (dextrorotary) or L (levorotary).

[0075] Polypeptides and polypeptide fragments sometimes are differentially modified during or after translation, *e.g.*, by glycosylation, acetylation, phosphorylation, amidation, derivatization by known protecting/blocking groups, proteolytic cleavage, linkage to an antibody molecule or other cellular ligand, etc. Any of numerous chemical modifications may be carried out by known techniques, including but not limited, to specific chemical cleavage by cyanogen bromide, trypsin, chymotrypsin, papain, V8 protease, NaBH4; acetylation, formylation, oxidation, reduction; metabolic synthesis in the presence of tunicamycin; and the like. Additional post-translational modifications include, for example, N-linked or O-linked carbohydrate chains, processing of N-terminal or C-terminal ends), attachment of chemical moieties to the amino acid backbone, chemical modifications of N-linked or O-linked carbohydrate chains, and addition or deletion of an N-terminal methionine residue as a result of procaryotic host cell expression. The polypeptide fragments may also be

modified with a detectable label, such as an enzymatic, fluorescent, isotopic or affinity label to allow for detection and isolation of the polypeptide.

[0076] Also provided are chemically modified derivatives of polypeptides that can provide additional advantages such as increased solubility, stability and circulating time of the polypeptide, or decreased immunogenicity (*see e.g.*, U.S. Pat. No: 4,179,337. The chemical moieties for derivitization may be selected from water soluble polymers such as polyethylene glycol, ethylene glycol/propylene glycol copolymers, carboxymethylcellulose, dextran, polyvinyl alcohol and the like. The polypeptides may be modified at random positions within the molecule, or at predetermined positions within the molecule and may include one, two, three or more attached chemical moieties.

[0077] The polymer may be of any molecular weight, and may be branched or unbranched. For polyethylene glycol, the molecular weight often utilized is between about 1 kDa and about 100 kDa (the term "about" indicating that in preparations of polyethylene glycol, some molecules will weigh more, some less, than the stated molecular weight) for ease in handling and manufacturing. Other sizes may be used, depending on the desired therapeutic profile (e.g., the duration of sustained release desired, the effects, if any on biological activity, the ease in handling, the degree or lack of antigenicity and other known effects of the polyethylene glycol to a therapeutic protein or analog).

[0078] The polymers should be attached to the polypeptide with consideration of effects on functional or antigenic domains of the polypeptide. There are a number of attachment methods available to those skilled in the art (*e.g.*, EP 0 401 384 (coupling PEG to G-CSF) and Malik et al. (1992) Exp Hematol. September;20(8):1028-35 (pegylation of GM-CSF using tresyl chloride)). For example, polyethylene glycol may be covalently bound through amino acid residues via a reactive group, such as a free amino or carboxyl group. Reactive groups are those to which an activated polyethylene glycol molecule may be bound. The amino acid residues having a free amino group may include lysine residues and the N-terminal amino acid residues; those having a free carboxyl group may include aspartic acid residues, glutamic acid residues and the C-terminal amino acid residue. Sulfhydryl groups may also be used as a reactive group for attaching the polyethylene glycol molecules. For therapeutic purposes, the attachment sometimes is at an amino group, such as attachment at the N-terminus or lysine group.

[0079] Proteins can be chemically modified at the N-terminus. Using polyethylene glycol as an illustration of such a composition, one may select from a variety of polyethylene glycol molecules (by molecular weight, branching, etc.), the proportion of polyethylene glycol molecules to protein (polypeptide) molecules in the reaction mix, the type of pegylation reaction to be performed, and the method of obtaining the selected N-terminally pegylated protein. The method of obtaining the N-terminally pegylated preparation (i.e., separating this moiety from other monopegylated moieties if necessary) may be by purification of the N-terminally pegylated material from a population of pegylated protein molecules. Selective proteins chemically modified at the N-terminus may be

accomplished by reductive alkylation, which exploits differential reactivity of different types of primary amino groups (lysine versus the N-terminal) available for derivatization in a particular protein. Under the appropriate reaction conditions, substantially selective derivatization of the protein at the N-terminus with a carbonyl group containing polymer is achieved.

Substantially Identical *BRAF* Nucleic Acids and Polypeptides

[0080] *BRAF* nucleotide sequences and *BRAF* polypeptide sequences that are substantially identical to the nucleotide sequence of Figure 1 and the polypeptide sequences of Figures 2A-2G, respectively, are included herein. The term "substantially identical" as used herein refers to two or more nucleic acids or polypeptides sharing one or more identical nucleotide sequences or polypeptide sequences, respectively. Included are nucleotide sequences or polypeptide sequences that are 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95% or more (each often within a 1%, 2%, 3% or 4% variability) identical to the *BRAF* nucleotide sequence in Figure 1 (SEQ ID NO: 1) or the *BRAF* polypeptide sequences of Figures 2A-2G (SEQ ID NO:). One test for determining whether two nucleic acids are substantially identical is to determine the percent of identical nucleotide sequences or polypeptide sequences shared between the nucleic acids or polypeptides.

[0081] Calculations of sequence identity are often performed as follows. Sequences are aligned for optimal comparison purposes (*e.g.*, gaps can be introduced in one or both of a first and a second amino acid or nucleic acid sequence for optimal alignment and non-homologous sequences can be disregarded for comparison purposes). The length of a reference sequence aligned for comparison purposes is sometimes 30% or more, 40% or more, 50% or more, often 60% or more, and more often 70%, 80%, 90%, 100% of the length of the reference sequence. The nucleotides or amino acids at corresponding nucleotide or polypeptide positions, respectively, are then compared among the two sequences. When a position in the first sequence is occupied by the same nucleotide or amino acid as the corresponding position in the second sequence, the nucleotides or amino acids are deemed to be identical at that position. The percent identity between the two sequences is a function of the number of identical positions shared by the sequences, taking into account the number of gaps, and the length of each gap, introduced for optimal alignment of the two sequences.

[0082] Comparison of sequences and determination of percent identity between two sequences can be accomplished using a mathematical algorithm. Percent identity between two amino acid or nucleotide sequences can be determined using the algorithm of Meyers & Miller, *CABIOS 4:* 11-17 (1989), which has been incorporated into the ALIGN program (version 2.0), using a PAM120 weight residue table, a gap length penalty of 12 and a gap penalty of 4. Also, percent identity between two amino acid sequences can be determined using the Needleman & Wunsch, *J. Mol. Biol. 48:* 444-453 (1970) algorithm which has been incorporated into the GAP program in the GCG software package (available at the http address www.gcg.com), using either a Blossum 62 matrix or a PAM250 matrix,

and a gap weight of 16, 14, 12, 10, 8, 6, or 4 and a length weight of 1, 2, 3, 4, 5, or 6. Percent identity between two nucleotide sequences can be determined using the GAP program in the GCG software package (available at http address www.gcg.com), using a NWSgapdna.CMP matrix and a gap weight of 40, 50, 60, 70, or 80 and a length weight of 1, 2, 3, 4, 5, or 6. A set of parameters often used is a Blossum 62 scoring matrix with a gap open penalty of 12, a gap extend penalty of 4, and a frameshift gap penalty of 5.

[0083] Another manner for determining if two nucleic acids are substantially identical is to assess whether a polynucleotide homologous to one nucleic acid will hybridize to the other nucleic acid under stringent conditions. As use herein, the term "stringent conditions" refers to conditions for hybridization and washing. Stringent conditions are known to those skilled in the art and can be found in *Current Protocols in Molecular Biology*, John Wiley & Sons, N.Y., 6.3.1-6.3.6 (1989). Aqueous and non-aqueous methods are described in that reference and either can be used. An example of stringent hybridization conditions is hybridization in 6X sodium chloride/sodium citrate (SSC) at about 45□C, followed by one or more washes in 0.2X SSC, 0.1% SDS at 50°C. Another example of stringent hybridization conditions are hybridization in 6X sodium chloride/sodium citrate (SSC) at about 45□C, followed by one or more washes in 0.2X SSC, 0.1% SDS at 55°C. A further example of stringent hybridization conditions is hybridization in 6X sodium chloride/sodium citrate (SSC) at about 45□C, followed by one or more washes in 0.2X SSC, 0.1% SDS at 60°C. Often, stringent hybridization conditions are hybridization in 6X sodium chloride/sodium citrate (SSC) at about 45□C, followed by one or more washes in 0.2X SSC, 0.1% SDS at 65°C. More often, stringency conditions are 0.5M sodium phosphate, 7% SDS at 65°C, followed by one or more washes at 0.2X SSC, 1% SDS at 65°C.

[0084] An example of a substantially identical nucleotide sequence to SEQ ID NO: 1 is one that has a different nucleotide sequence and still encodes a polypeptide sequence set forth in Figures 2A-2G. Another example is a nucleotide sequence that encodes a polypeptide having a polypeptide sequence that is more than 70% identical to, sometimes more than 75%, 80%, or 85% identical to, and often more than 90% and 95% or more identical to the polypeptide sequences set forth in Figures 2A-2G.

[0085] *BRAF* nucleotide sequences and polypeptide sequences can be used as "query sequences" to perform a search against public databases to identify other family members or related sequences, for example. Such searches can be performed using the NBLAST and XBLAST programs (version 2.0) of Altschul *et al., J. Mol. Biol. 215:* 403-10 (1990). BLAST nucleotide searches can be performed with the NBLAST program, score = 100, wordlength = 12 to obtain nucleotide sequences homologous to *BRAF* nucleic acid molecules. BLAST polypeptide searches can be performed with the XBLAST program, score = 50, wordlength = 3 to obtain amino acid sequences homologous to *BRAF* polypeptides. To obtain gapped alignments for comparison purposes, Gapped BLAST can be

utilized as described in Altschul *et al., Nucleic Acids Res. 25(17):* 3389-3402 (1997). When utilizing BLAST and Gapped BLAST programs, default parameters of the respective programs (*e.g.*, XBLAST and NBLAST) can be used (*see* the http address www.ncbi.nlm.nih.gov).

[0086] A nucleic acid that is substantially identical to the nucleotide sequence of SEQ ID NO: 1 may include polymorphic sites at positions equivalent to those described herein (*e.g.*, position 146311 in SEQ ID NO: 1) when the sequences are aligned. For example, using the alignment procedures described herein, SNPs in a sequence substantially identical to the sequence of SEQ ID NO: 1 can be identified at nucleotide positions that match (*i.e.*, align) with nucleotides at SNP positions in SEQ ID NO: 1. Also, where a polymorphic variation is an insertion or deletion, insertion or deletion of a nucleotide sequence from a reference sequence can change the relative positions of other polymorphic sites in the nucleotide sequence.

[0087] Substantially identical *BRAF* nucleotide and polypeptide sequences include those that are naturally occurring, such as allelic variants (same locus), splice variants, homologs (different locus), and orthologs (different organism) or can be non-naturally occurring. Non-naturally occurring variants can be generated by mutagenesis techniques, including those applied to polynucleotides, cells, or organisms. The variants can contain nucleotide substitutions, deletions, inversions and insertions. Variation can occur in either or both the coding and non-coding regions. The variations can produce both conservative and non-conservative amino acid substitutions (as compared in the encoded product). Orthologs, homologs, allelic variants, and splice variants can be identified using methods known in the art. These variants normally comprise a nucleotide sequence encoding a polypeptide that is 50%, about 55% or more, often about 70-75% or more, more often about 80-85% or more, and typically about 90-95% or more identical to the amino acid sequences shown in Figures 2A-2G or a fragment thereof. Such nucleic acid molecules can readily be identified as being able to hybridize under stringent conditions to the nucleotide sequence shown in SEQ ID NO: 1 or a fragment of this sequence. Nucleic acid molecules corresponding to orthologs, homologs, and allelic variants of the *BRAF* nucleotide sequence can further be identified by mapping the sequence to the same chromosome or locus as the *BRAF* nucleotide sequence or variant.

[0088] Also, substantially identical *BRAF* nucleotide sequences may include codons that are altered with respect to the naturally occurring sequence for enhancing expression of a *BRAF* polypeptide or polypeptide variant in a particular expression system. For example, the nucleic acid can be one in which one or more codons are altered, and often 10% or more or 20% or more of the codons are altered for optimized expression in bacteria (*e.g.*, *E. coli*.), yeast (*e.g.*, *S. cervesiae*), human (*e.g.*, 293 cells), insect, or rodent (*e.g.*, hamster) cells.

Methods for Identifying Subjects at Risk of Melanoma

[0089] Methods for determining whether a subject is at risk of melanoma are provided herein. These methods include detecting the presence or absence of one or more polymorphic variations associated with melanoma in a *BRAF* nucleotide sequence, or substantially identical sequence thereof, in a sample from a subject, where the presence of such a polymorphic variation is indicative of the subject being at risk of melanoma. These genetic tests are useful for prognosing and/or diagnosing melanoma and often are useful for determining whether an individual is at an increased, intermediate or decreased risk of developing or having melanoma.

[0090] Thus, featured herein is a method for identifying a subject at risk of melanoma, which comprises detecting in a nucleic acid sample from the subject the presence or absence of a polymorphic variation associated with melanoma at a polymorphic site in a *BRAF* nucleotide sequence. The nucleotide sequence often is selected from the group consisting of: (a) a nucleotide sequence set forth in SEQ ID NO:1; (b) a nucleotide sequence which encodes a polypeptide consisting of an amino acid sequence described in Figures 2A to 2G or Figure 3B; (c) a nucleotide sequence which encodes a polypeptide that is 90% or more identical to an amino acid sequence described in Figures 2A to 2G or Figure 3B or a nucleotide sequence about 90% or more identical to the nucleotide sequence set forth in SEQ ID NO:1; and (d) a fragment of a nucleotide sequence of (a), (b), or (c), where the fragment comprises a polymorphic site; whereby the presence of the polymorphic variation is indicative of the subject being at risk of melanoma. A polymorphic variation assayed in the genetic test often is located in an intron, sometimes in a region surrounding the *BRAF* open reading frame (*e.g.*, within 50 kilobases (kb), 40 kb, 30 kb, 20 kb, 15, kb, 10 kb, 5 kb, 4 kb, 3 kb, 2 kb, or 1 kb of the open reading frame initiation site or termination site), and sometimes in an exon. Sometimes the polymorphic variation is not located in an exon. In embodiments where an exonic polymorphic variation is assayed, it often is located in an exon other than exon 15 of the *BRAF* nucleotide sequence; sometimes does not lead to an amino acid variation; often does not lead to an amino acid variation of valine 599 in Figure 3B; and often does not lead to a valine 599 to glutamate or a valine 599 to lysine amino acid modification in Figure 3B.

[0091] Results from such genetic tests may be combined with other test results to diagnose melanoma. For example, genetic test results may be gathered, a patient sample may be ordered based on a determined predisposition to melanoma (*e.g.*, a skin biopsy), the patient sample is analyzed, and the results of the analysis may be utilized to diagnose melanoma. Also, melanoma diagnostic tests are generated by stratifying populations into subpopulations having different progressions of melanoma and detecting polymorphic variations associated with different progressions of the melanoma, as described in further detail hereafter. In another embodiment, genetic test results are gathered, a patient's risk factors for developing melanoma are analyzed (*e.g.*, exposure to sun and skin pigmentation), and a patient sample may be ordered based on a determined risk of melanoma.

[0092] Risk of melanoma sometimes is expressed as a probability, such as an odds ratio, percentage, or risk factor. The risk assessment is based upon the presence or absence of one or more polymorphic variants described herein, and also may be based in part upon phenotypic traits of the individual being tested. Methods for calculating risks based upon patient data are well known (*see, e.g.*, Agresti, *Categorical Data Analysis*, 2nd Ed. 2002. Wiley). Allelotyping and genotyping analyses may be carried out in populations other than those exemplified herein to enhance the predictive power of the prognostic method. These further analyses are executed in view of the exemplified procedures described herein, and may be based upon the same polymorphic variations or additional polymorphic variations.

[0093] The nucleic acid sample typically is isolated from a biological sample obtained from a subject. For example, nucleic acid can be isolated from blood, saliva, sputum, urine, cell scrapings, and biopsy tissue. The nucleic acid sample can be isolated from a biological sample using standard techniques, such as the technique described in Example 2. As used herein, the term "subject" refers primarily to humans but also refers to other mammals such as dogs, cats, and ungulates (*e.g.*, cattle, sheep, and swine). Subjects also include avians (*e.g.*, chickens and turkeys), reptiles, and fish (*e.g.*, salmon), as embodiments described herein can be adapted to nucleic acid samples isolated from any of these organisms. The nucleic acid sample may be isolated from the subject and then directly utilized in a method for determining the presence of a polymorphic variant, or alternatively, the sample may be isolated and then stored (*e.g.*, frozen) for a period of time before being subjected to analysis.

[0094] The presence or absence of a polymorphic variant is determined using one or both chromosomal complements represented in the nucleic acid sample. Determining the presence or absence of a polymorphic variant in both chromosomal complements represented in a nucleic acid sample from a subject having a copy of each chromosome is useful for determining the zygosity of an individual for the polymorphic variant (*i.e.*, whether the individual is homozygous or heterozygous for the polymorphic variant). Any oligonucleotide-based diagnostic may be utilized to determine whether a sample includes the presence or absence of a polymorphic variant in a sample. For example, primer extension methods, ligase sequence determination methods (*e.g.*, U.S. Pat. Nos. 5,679,524 and 5,952,174, and WO 01/27326), mismatch sequence determination methods (*e.g.*, U.S. Pat. Nos. 5,851,770; 5,958,692; 6,110,684; and 6,183,958), microarray sequence determination methods, restriction fragment length polymorphism (RFLP), single strand conformation polymorphism detection (SSCP) (*e.g.*, U.S. Pat. Nos. 5,891,625 and 6,013,499), PCR-based assays (*e.g.*, TAQMAN® PCR System (Applied Biosystems)), and nucleotide sequencing methods may be used.

[0095] Oligonucleotide extension methods typically involve providing a pair of oligonucleotide primers in a polymerase chain reaction (PCR) or in other nucleic acid amplification methods for the purpose of amplifying a region from the nucleic acid sample that comprises the

polymorphic variation. One oligonucleotide primer is complementary to a region 3' of the polymorphism and the other is complementary to a region 5' of the polymorphism. A PCR primer pair may be used in methods disclosed in U.S. Pat. Nos. 4,683,195; 4,683,202, 4,965,188; 5,656,493; 5,998,143; 6,140,054; WO 01/27327; and WO 01/27329 for example. PCR primer pairs may also be used in any commercially available machines that perform PCR, such as any of the GENEAMP® Systems available from Applied Biosystems. Also, those of ordinary skill in the art will be able to design oligonucleotide primers based upon the nucleotide sequence of SEQ ID NO: 1 without undue experimentation using knowledge readily available in the art.

[0096] Also provided are extension oligonucleotides that hybridize to the amplified fragment adjacent to the polymorphic variation. As used herein, the term "adjacent" refers to the 3' end of the extension oligonucleotide being sometimes 1 nucleotide from the 5' end of the polymorphic site, often 2 or 3, and at times 4, 5, 6, 7, 8, 9, or 10 nucleotides from the 5' end of the polymorphic site, in the nucleic acid when the extension oligonucleotide is hybridized to the nucleic acid. The extension oligonucleotide then is extended by one or more nucleotides, often 2 or 3 nucleotides, and the number and/or type of nucleotides that are added to the extension oligonucleotide determine whether the polymorphic variant is present. Oligonucleotide extension methods are disclosed, for example, in U.S. Pat. Nos. 4,656,127; 4,851,331; 5,679,524; 5,834,189; 5,876,934; 5,908,755; 5,912,118; 5,976,802; 5,981,186; 6,004,744; 6,013,431; 6,017,702; 6,046,005; 6,087,095; 6,210,891; and WO 01/20039. Oligonucleotide extension methods using mass spectrometry are described, for example, in U.S. Pat. Nos. 5,547,835; 5,605,798; 5,691,141; 5,849,542; 5,869,242; 5,928,906; 6,043,031; and 6,194,144, and a method often utilized is described herein in Example 2.

[0097] A microarray can be utilized for determining whether a polymorphic variant is present or absent in a nucleic acid sample. A microarray may include any oligonucleotides described herein, and methods for making and using oligonucleotide microarrays suitable for prognostic use are disclosed in U.S. Pat. Nos. 5,492,806; 5,525,464; 5,589,330; 5,695,940; 5,849,483; 6,018,041; 6,045,996; 6,136,541; 6,142,681; 6,156,501; 6,197,506; 6,223,127; 6,225,625; 6,229,911; 6,239,273; WO 00/52625; WO 01/25485; and WO 01/29259. The microarray typically comprises a solid support and the oligonucleotides may be linked to this solid support by covalent bonds or by non-covalent interactions. The oligonucleotides may also be linked to the solid support directly or by a spacer molecule. A microarray may comprise one or more oligonucleotides complementary to a polymorphic site of SEQ ID NO: 1.

[0098] A kit may also be utilized for determining whether a polymorphic variant is present or absent in a nucleic acid sample. A kit often comprises one or more pairs of oligonucleotide primers useful for amplifying a fragment of SEQ ID NO: 1 or a substantially identical sequence thereof, where the fragment includes a polymorphic site. The kit sometimes comprises a polymerizing agent, for example, a thermostable nucleic acid polymerase such as one disclosed in U.S. Pat. Nos. 4,889,818 or

6,077,664. Also, the kit often comprises an elongation oligonucleotide that hybridizes to a *BRAF* nucleic acid in a nucleic acid sample adjacent to the polymorphic site. Where the kit includes an elongation oligonucleotide, it also often comprises chain elongating nucleotides, such as dATP, dTTP, dGTP, dCTP, and dITP, including analogs of dATP, dTTP, dGTP, dCTP and dITP, provided that such analogs are substrates for a thermostable nucleic acid polymerase and can be incorporated into a nucleic acid chain elongated from the extension oligonucleotide. Along with chain elongating nucleotides would be one or more chain terminating nucleotides such as ddATP, ddTTP, ddGTP, ddCTP, and the like. In an embodiment, the kit comprises one or more oligonucleotide primer pairs, a polymerizing agent, chain elongating nucleotides, at least one elongation oligonucleotide, and one or more chain terminating nucleotides. Kits optionally include buffers, vials, microtitre plates, and instructions for use. *BRAF* directed hits may be utilized to prognose or diagnose melanoma for a significant fraction of melanoma occurrences, such as in 50% or more melanoma occurrences, or sometimes 60% or more, 70% or more, or 80% or more.

[0099] Using a polymorphism detection technology (*e.g.*, a technique described above or below in Example 2), mutations and polymorphisms in or around the *BRAF* locus may be detected in melanocytic lesions, which include nevi, radial growth phase (RGP) melanomas, vertical growth phase (VGP) melanomas, and melanoma metastases. The mutations can be detected within 50 kilobases (kb), 40 kb, 30 kb, 20 kb, 15, kb, 10 kb, 5 kb, 4 kb, 3 kb, 2 kb, or 1 kb from the BRAF open reading frame initiation or termination site. Therefore, provided herein are methods for genotyping *BRAF* mutations in melanocytic lesions and metastases (*e.g.*, described in Example 2). Mutations in or around the BRAF locus present in later stage melanomas, such as VGP melanomas and melanoma metastases, are indicative of melanomas particularly likely to continue to progress and/or metastasize (*e.g.*, from RGP to VGP melanoma or melanoma metastases), *i.e.*, aggressive melanomas. Thus, provided herein are methods for identifying subjects at risk of a progressive or aggressive melanoma by determining the presence or absence of one or more *BRAF* mutations in the DNA sample of a subject that exist in melanocytic lesions and/or metastases. Identifying the presence of one or more of these mutations is useful for identifying subjects in need of aggressive treatments of melanoma, and once identified using such methods, a subject often is given information concerning preventions and treatments of the disease, and sometimes is treated with an aggressive melanoma treatment method (*e.g.*, surgery or administration of drugs), as described in more detail hereafter.

[00100] Determining the presence of a polymorphic variant, or a combination of two or more polymorphic variants, in a nucleic acid set forth in SEQ ID NO:1 of the sample is often indicative of a predisposition to melanoma. For example, the presence of the haplotype CTTG in males or females, or the haplotype ATGA in males, at positions 146311, 138875, 76779, and 68398, respectively, in the reverse strand of a *BRAF* nucleotide sequence (SEQ ID NO: 1) are associated with an increased risk of melanoma. Similarly, the presence of the haplotype GAAC in males or females, or the haplotype

TACT, at positions 146311, 138875, 76779, and 68398, respectively, in the strand complementary to the sense strand of a *BRAF* nucleotide sequence (*i.e.*, the forward strand that is complementary to the strand reported in Figure 1) are associated with an increased risk of melanoma. Also, predisposition to melanoma is associated in males and females having the haplotype GGTTCGCATACT or GGTTCGTATATC, in females having the haplotype GATTCGCATACC, and in males having the haplotype TACCGATCCCTT (each haplotype corresponds to positions 146311, 138875, 132526, 128002, 118712, 98846, 98682, 87826, 80400, 76779, 68398 and 64547, respectively, of SEQ ID NO: 1, and is reported in the forward orientation of the *BRAF* gene (complementary to the sequence in Figure 1)). Similarly, predisposition to melanoma is associated in males and females having the haplotype CCAAGCGTATGA or CCAAGCATATAG, in females having the haplotype CTAAGCGTATGG, and in males having the haplotype ATGGCTAGGGAA (each twelve-position haplotype corresponds to positions 146311, 138875, 132526, 128002, 118712, 98846, 98682, 87826, 80400, 76779, 68398 and 64547, respectively, of SEQ ID NO: 1, and is reported in the reverse orientation of the *BRAF* gene (the sequence in Figure 1)). An individual identified as having a predisposition to melanoma may be heterozygous or homozygous with respect to the allele associated with melanoma.

[00101] Also, the presence of a thymine at position 146311 in the reverse strand of a *BRAF* nucleotide sequence in males is associated with an increased risk of melanoma. Similarly, the presence of an adenine at position 146311 in the strand complementary to the reverse strand of a *BRAF* nucleotide sequence, the forward strand, in males is associated with an increased risk of melanoma. An individual identified as having a predisposition to melanoma may be heterozygous or homozygous with respect to the allele associated with melanoma.

Applications of Prognostic Results to Pharmacogenomics

[00102] Pharmacogenomics is a discipline that involves tailoring a treatment for a subject according to the subject's genotype as a particular treatment regimen may exert a differential effect depending upon the subject's genotype. Based upon the outcome of a prognostic test described herein, a clinician or physician may target pertinent information and preventative or therapeutic treatments to a subject who would be benefited by the information or treatment and avoid directing such information and treatments to a subject who would not be benefited (*e.g.*, the treatment has no therapeutic effect and/or the subject experiences adverse side effects).

[00103] For example, where a candidate therapeutic exhibits a significant interaction with a major allele and a comparatively weak interaction with a minor allele (*e.g.*, an order of magnitude or greater difference in the interaction), such a therapeutic typically would not be administered to a subject genotyped as being homozygous for the minor allele, and sometimes not administered to a subject genotyped as being heterozygous for the minor allele. In another example, where a candidate

therapeutic is not significantly toxic when administered to subjects who are homozygous for a major allele but is comparatively toxic when administered to subjects heterozygous or homozygous for a minor allele, the candidate therapeutic is not typically administered to subjects who are genotyped as being heterozygous or homozygous with respect to the minor allele.

[00104] The prognostic methods described herein are applicable to general pharmacogenomic approaches towards addressing melanoma. For example, a nucleic acid sample from an individual may be subjected to a prognostic test described herein. Where one or more polymorphic variations associated with increased risk of melanoma are identified in a subject, one or more melanoma treatments or prophylactic regimens may be prescribed to that subject. For example, a male or female having the haplotype CTTG (corresponding to positions 146311, 138875, 76779, and 68398, respectively, in SEQ ID NO: 1), a male having the haplotype ATGA (same nomenclature as for the CTTG haplotype), or a male having an adenine at position 146311 of SEQ ID NO: 1 typically would be prescribed a prophylactic regimen designed to minimize the occurance of melanoma. Also, a male or female having the haplotype GGTTCGCATACT or GGTTCGTATATC, a female having the haplotype GATTCGCATACC, or a male having the haplotype TACCGATCCCTT (each twelve-position haplotype corresponds to positions 146311, 138875, 132526, 128002, 118712, 98846, 98682, 87826, 80400, 76779, 68398 and 64547, respectively, of SEQ ID NO: 1, and is reported in the forward orientation of the *BRAF* gene (complementary to the sequence in Figure 1)) typically would be prescribed a prophylactic regimen designed to minimize the occurance of melanoma. An example of a prophylactic regimen often prescribed is directed towards minimizing ultraviolet (UV) light exposure. Such a regimen may include, for example, prescription of a lotion applied to the skin that minimizes UV penetration and/or counseling individuals of other practices for reducing UV exposure, such as by wearing protective clothing and minimizing sun exposure.

[00105] In certain embodiments, a treatment regimen is specifically prescribed and/or administered to individuals who will most benefit from it based upon their risk of developing melanoma assessed by the prognostic methods described herein. Thus, provided are methods for identifying a subject predisposed to melanoma and then prescribing a therapeutic or preventative regimen to individuals identified as having a predisposition. Thus, certain embodiments are directed to a method for reducing melanoma in a subject, which comprises: detecting the presence or absence of a polymorphic variant associated with melanoma in a nucleotide sequence set forth in Figure 1 in a nucleic acid sample from a subject, where the nucleotide sequence comprises a polynucleotide sequence selected from the group consisting of: (a) a nucleotide sequence set forth in Figure 1; (b) a nucleotide sequence which encodes a polypeptide consisting of an amino acid sequence described in Figure 1; (c) a nucleotide sequence which encodes a polypeptide that is 90% or more identical to an amino acid sequence described in Figure 1 or a nucleotide sequence about 90% or more identical to the nucleotide sequence set forth in Figure 1; and (d) a fragment of a polynucleotide sequence of (a),

(b), or (c); and prescribing or administering a treatment regimen to a subject from whom the sample originated where the presence of a polymorphic variation associated with melanoma is detected in the nucleotide sequence. In these methods, predisposition results may be utilized in combination with other test results to diagnose melanoma.

[00106] The treatment sometimes is preventative (*e.g.*, is prescribed or administered to reduce the probability that a melanoma associated condition arises or progresses), sometimes is therapeutic, and sometimes delays, alleviates or halts the progression of a melanoma associated condition. Any known preventative or therapeutic treatment for alleviating or preventing the occurrence of a melanoma associated disorder is prescribed and/or administered. For example, the treatment sometimes is or includes a drug that reduces melanoma, including, for example, cisplatin, carmustine (BCNU), vinblastine, vincristine, and bleomycin, and/or a molecule that interacts with a nucleic acid or polypeptide described hereafter. In another example, the melanoma treatment is surgery. Surgery to remove (excise) a melanoma is the standard treatment for this disease. It is necessary to remove not only the tumor but also some normal tissue around it in order to minimize the chance that any cancer will be left in the area. It is common for lymph nodes near the tumor to be removed during surgery because cancer can spread through the lymphatic system. Surgery is generally not effective in controlling melanoma that is known to have spread to other parts of the body. In such cases, doctors may use other methods of treatment, such as chemotherapy, biological therapy, radiation therapy, or a combination of these methods.

[00107] As therapeutic approaches for melanoma continue to evolve and improve, the goal of treatments for melanoma related disorders is to intervene even before clinical signs (*e.g.*, identification of irregular nevi based on A- asymmetry, B- border irregularity, C- color variation, D- diameter of > 6 mm as described by Friedman RJ, et al. in *CA Cancer J Clin.* 1985 May-Jun;35(3):130-51) first manifest. Thus, genetic markers associated with susceptibility to melanoma prove useful for early diagnosis, prevention and treatment of melanoma.

[00108] As melanoma preventative and treatment information can be specifically targeted to subjects in need thereof (*e.g.*, those at risk of developing melanoma or those that have early signs of melanoma), provided herein is a method for preventing or reducing the risk of developing melanoma in a subject, which comprises: (a) detecting the presence or absence of a polymorphic variation associated with melanoma at a polymorphic site in a nucleotide sequence in a nucleic acid sample from a subject; (b) identifying a subject with a predisposition to melanoma, whereby the presence of the polymorphic variation is indicative of a predisposition to melanoma in the subject; and (c) if such a predisposition is identified, providing the subject with information about methods or products to prevent or reduce melanoma or to delay the onset of melanoma. Also provided is a method of targeting information or advertising to a subpopulation of a human population based on the subpopulation being genetically predisposed to a disease or condition, which comprises: (a) detecting

the presence or absence of a polymorphic variation associated with melanoma at a polymorphic site in a nucleotide sequence in a nucleic acid sample from a subject; (b) identifying the subpopulation of subjects in which the polymorphic variation is associated with melanoma; and (c) providing information only to the subpopulation of subjects about a particular product which may be obtained and consumed or applied by the subject to help prevent or delay onset of the disease or condition.

[00109] Pharmacogenomics methods also may be used to analyze and predict a response to a melanoma treatment or a drug. For example, if pharmacogenomics analysis indicates a likelihood that an individual will respond positively to a melanoma treatment with a particular drug, the drug may be administered to the individual. Conversely, if the analysis indicates that an individual is likely to respond negatively to treatment with a particular drug, an alternative course of treatment may be prescribed. A negative response may be defined as either the absence of an efficacious response or the presence of toxic side effects. The response to a therapeutic treatment can be predicted in a background study in which subjects in any of the following populations are genotyped: a population that responds favorably to a treatment regimen, a population that does not respond significantly to a treatment regimen, and a population that responds adversely to a treatment regiment (*e.g.*, exhibits one or more side effects). These populations are provided as examples and other populations and subpopulations may be analyzed. Based upon the results of these analyses, a subject is genotyped to predict whether he or she will respond favorably to a treatment regimen, not respond significantly to a treatment regimen, or respond adversely to a treatment regimen.

[00110] The prognostic tests described herein also are applicable to clinical drug trials. One or more polymorphic variants indicative of response to an agent for treating melanoma or to side effects to an agent for treating melanoma may be identified using the methods described herein. Thereafter, potential participants in clinical trials of such an agent may be screened to identify those individuals most likely to respond favorably to the drug and exclude those likely to experience side effects. In that way, the effectiveness of drug treatment may be measured in individuals who respond positively to the drug, without lowering the measurement as a result of the inclusion of individuals who are unlikely to respond positively in the study and without risking undesirable safety problems.

[00111] Thus, another embodiment is a method of selecting an individual for inclusion in a clinical trial of a treatment or drug comprising the steps of: (a) obtaining a nucleic acid sample from an individual; (b) determining the identity of a polymorphic variation which is associated with a positive response to the treatment or the drug, or at least one polymorphic variation which is associated with a negative response to the treatment or the drug in the nucleic acid sample, and (c) including the individual in the clinical trial if the nucleic acid sample contains said polymorphic variation associated with a positive response to the treatment or the drug or if the nucleic acid sample lacks said polymorphic variation associated with a negative response to the treatment or the drug. In addition, the methods for selecting an individual for inclusion in a clinical trial of a treatment or drug

encompass methods with any further limitation described in this disclosure, or those following, specified alone or in any combination. The polymorphic variation may be in a sequence selected individually or in any combination from the group consisting of (i) a polynucleotide sequence set forth in Figure: 1; (ii) a polynucleotide sequence that is 90% or more identical to a nucleotide sequence set forth in Figure 1; (iii) a polynucleotide sequence that encodes a polypeptide having an amino acid sequence identical to or 90% or more identical to an amino acid sequence encoded by a nucleotide sequence set forth in Figure 1; and (iv) a fragment of a polynucleotide sequence of (i), (ii), or (iii) comprising the polymorphic site. The including step (c) optionally comprises administering the drug or the treatment to the individual if the nucleic acid sample contains the polymorphic variation associated with a positive response to the treatment or the drug and the nucleic acid sample lacks said biallelic marker associated with a negative response to the treatment or the drug.

[00112] Also provided herein is a method of partnering between a diagnostic/prognostic testing provider and a provider of a consumable product, which comprises: (a) the diagnostic/prognostic testing provider detects the presence or absence of a polymorphic variation associated with melanoma at a polymorphic site in a nucleotide sequence in a nucleic acid sample from a subject; (b) the diagnostic/prognostic testing provider identifies the subpopulation of subjects in which the polymorphic variation is associated with melanoma; (c) the diagnostic/prognostic testing provider forwards information to the subpopulation of subjects about a particular product which may be obtained and consumed or applied by the subject to help prevent or delay onset of the disease or condition; and (d) the provider of a consumable product forwards to the diagnostic test provider a fee every time the diagnostic/prognostic test provider forwards information to the subject as set forth in step (c) above.

Methods for Identifying Candidate Therapeutics for Treating Melanoma

[00113] Featured herein are methods for identifying a candidate therapeutic for treating melanoma The methods comprise contacting a test molecule with a *BRAF* nucleic acid, substantially identical nucleic acid, polypeptide, or substantially identical polypeptide in a system. The nucleic acid is often the *BRAF* nucleotide sequence represented by SEQ ID NO: 1, sometimes a nucleotide sequence that is substantially identical to the nucleotide sequence of SEQ ID NO: 1, or sometimes a fragment thereof, and the *BRAF* polypeptide is a polypeptide encoded by any of these nucleic acids. The method also comprises determining the presence or absence of an interaction between the test molecule and the *BRAF* nucleic acid or polypeptide, where the presence of an interaction between the test molecule and the *BRAF* nucleic acid or polypeptide identifies the test molecule as a candidate melanoma therapeutic.

[00114] As used herein, the term "test molecule" and "candidate therapeutic" refers to modulators of regulation of transcription and translation of *BRAF* nucleic acids and modulations of

expression and activity of *BRAF* polypeptides. The term "modulator" as used herein refers to a molecule which agonizes or antagonizes *BRAF* DNA replication and/or DNA processing (*e.g.*, methylation), *BRAF* RNA transcription and/or RNA processing (*e.g.*, removal of intronic sequences and/or translocation from the nucleus), *BRAF* polypeptide production (*e.g.*, translation of the polypeptide from mRNA, and/or post-translational modification such as glycosylation, phosphorylation, and proteolysis of pro-polypeptides), and/or *BRAF* function (*e.g.*, conformational changes, binding of nucleotides or nucleotide analogs, interaction with binding partners, effect on phosphorylation, and/or effect on occurrence of melanoma). Test molecules and candidate therapeutics include, but are not limited to, compounds, siRNA molecules, antisense nucleic acids, ribozymes, *BRAF* polypeptide or fragments thereof, immunotherapeutics (*e.g.*, antibodies and HLA-presented polypeptide fragments).

Compounds

[00115] Compounds may be utilized as test molecules for identifying candidate therapeutics for treating melanoma. Compounds can be obtained using any of the numerous approaches in combinatorial library methods known in the art, including: biological libraries; peptoid libraries (libraries of molecules having the functionalities of peptides, but with a novel, non-peptide backbone which are resistant to enzymatic degradation but which nevertheless remain bioactive (see, *e.g.*, Zuckermann *et al., J. Med. Chem.37:* 2678-85 (1994)); spatially addressable parallel solid phase or solution phase libraries; synthetic library methods requiring deconvolution; "one-bead one-compound" library methods; and synthetic library methods using affinity chromatography selection. Biological library and peptoid library approaches are typically limited to peptide libraries, while the other approaches are applicable to peptide, non-peptide oligomer or small molecule libraries of compounds (Lam, *Anticancer Drug Des. 12:* 145, (1997)). Examples of methods for synthesizing molecular libraries are described, for example, in DeWitt *et al., Proc. Natl. Acad. Sci. U.S.A. 90:* 6909 (1993); Erb *et al., Proc. Natl. Acad. Sci. USA 91:* 11422 (1994); Zuckermann *et al., J. Med. Chem. 37:* 2678 (1994); Cho *et al., Science 261:* 1303 (1993); Carrell *et al., Angew. Chem. Int. Ed. Engl. 33:* 2059 (1994); Carell *et al., Angew. Chem. Int. Ed. Engl. 33*: 2061 (1994); and in Gallop *et al., J. Med. Chem. 37:* 1233 (1994).

[00116] Libraries of compounds may be presented in solution (*e.g.*, Houghten, *Biotechniques 13:* 412-421 (1992)), or on beads (Lam, *Nature 354:* 82-84 (1991)), chips (Fodor, *Nature 364:* 555-556 (1993)), bacteria or spores (Ladner, United States Patent No. 5,223,409), plasmids (Cull *et al., Proc. Natl. Acad. Sci. USA 89:* 1865-1869 (1992)) or on phage (Scott and Smith, *Science 249:* 386-390 (1990); Devlin, *Science 249:* 404-406 (1990); Cwirla *et al., Proc. Natl. Acad. Sci. 87:* 6378-6382 (1990); Felici, *J. Mol. Biol. 222:* 301-310 (1991); Ladner *supra.*).

[00117] Compounds may alter expression or activity of *BRAF* polypeptides and may be a small molecule. Small molecules include, but are not limited to, peptides, peptidomimetics (*e.g.*, peptoids), amino acids, amino acid analogs, polynucleotides, polynucleotide analogs, nucleotides, nucleotide analogs, organic or inorganic compounds (*i.e.*, including heteroorganic and organometallic compounds) having a molecular weight less than about 10,000 grams per mole, organic or inorganic compounds having a molecular weight less than about 5,000 grams per mole, organic or inorganic compounds having a molecular weight less than about 1,000 grams per mole, organic or inorganic compounds having a molecular weight less than about 500 grams per mole, and salts, esters, and other pharmaceutically acceptable forms of such compounds.

Antisense Nucleic Acid Molecules, Ribozymes, and Modified *BRAF* Nucleic Acid Molecules

[00118] Also featured herein are antisense, ribozyme, and modified *BRAF* nucleic acids for use as test molecules in methods for identifying candidate therapeutics for treating melanoma and for use as therapeutics for treating melanoma in a subject. An "antisense" nucleic acid refers to a nucleotide sequence which is complementary to a "sense" nucleic acid encoding a polypeptide, *e.g.*, complementary to the coding strand of a double-stranded cDNA molecule or complementary to an mRNA sequence. The antisense nucleic acid can be complementary to an entire *BRAF* coding strand, or to only a portion thereof (*e.g.*, the coding region of human *BRAF* corresponding to SEQ ID NO: 1). In another embodiment, the antisense nucleic acid molecule is antisense to a "noncoding region" of the coding strand of a nucleotide sequence encoding *BRAF* (*e.g.*, 5' and 3' untranslated regions).

[00119] An antisense nucleic acid can be designed such that it is complementary to the entire coding region of *BRAF* mRNA, and often the antisense nucleic acid is an oligonucleotide that is antisense to only a portion of a coding or noncoding region of *BRAF* mRNA. For example, the antisense oligonucleotide can be complementary to the region surrounding the translation start site of *BRAF* mRNA, *e.g.*, between the -10 and +10 regions of the target gene nucleotide sequence of interest. An antisense oligonucleotide can be, for example, about 7, 10, 15, 20, 25, 30, 35, 40, 45, 50, 55, 60, 65, 70, 75, 80, or more nucleotides in length. The antisense nucleic acids, which include the ribozymes described hereafter, can be designed to target *BRAF* nucleic acid or *BRAF* nucleic acid variants. Among the variants, minor alleles and major alleles can be targeted, and those associated with a higher risk of melanoma are often designed, tested, and administered to subjects.

[00120] An antisense nucleic acid can be constructed using chemical synthesis and enzymatic ligation reactions using standard procedures. For example, an antisense nucleic acid (*e.g.*, an antisense oligonucleotide) can be chemically synthesized using naturally occurring nucleotides or variously modified nucleotides designed to increase the biological stability of the molecules or to increase the physical stability of the duplex formed between the antisense and sense nucleic acids, *e.g.*, phosphorothioate derivatives and acridine substituted nucleotides can be used. Antisense nucleic

acid also can be produced biologically using an expression vector into which a nucleic acid has been subcloned in an antisense orientation (*i.e.*, RNA transcribed from the inserted nucleic acid will be of an antisense orientation to a target nucleic acid of interest, described further in the following subsection).

[00121] Antisense nucleic acids are typically administered to a subject (*e.g.*, by direct injection at a tissue site) or generated *in situ* such that they hybridize with or bind to cellular mRNA and/or genomic DNA encoding a *BRAF* polypeptide and thereby inhibit expression of the polypeptide, for example, by inhibiting transcription and/or translation. Alternatively, antisense nucleic acid molecules can be modified to target selected cells and then administered systemically. For systemic administration, antisense molecules can be modified such that they specifically bind to receptors or antigens expressed on a selected cell surface, for example, by linking antisense nucleic acid molecules to peptides or antibodies which bind to cell surface receptors or antigens. Antisense nucleic acid molecules can also be delivered to cells using the vectors described herein. Sufficient intracellular concentrations of antisense molecules are achieved by incorporating a strong promoter, such as a pol II or pol III promoter, in the vector construct.

[00122] Antisense nucleic acid molecules are sometimes α-anomeric nucleic acid molecules. An α-anomeric nucleic acid molecule forms specific double-stranded hybrids with complementary RNA in which, contrary to the usual β-units, the strands run parallel to each other (Gaultier *et al., Nucleic Acids. Res. 15:* 6625-6641 (1987)). Antisense nucleic acid molecules can also comprise a 2'-o-methylribonucleotide (Inoue *et al., Nucleic Acids Res. 15:* 6131-6148 (1987)) or a chimeric RNA-DNA analogue (Inoue *et al., FEBS Lett. 215:* 327-330 (1987)).

[00123] In another embodiment, an antisense nucleic acid is a ribozyme. A ribozyme having specificity for a *BRAF*-encoding nucleic acid can include one or more sequences complementary to the nucleotide sequence of a *BRAF* DNA sequence disclosed herein (*e.g.*, SEQ ID NO: 1), and a sequence having a known catalytic sequence responsible for mRNA cleavage (see U.S. Pat. No. 5,093,246 or Haselhoff and Gerlach, *Nature 334:* 585-591 (1988)). For example, a derivative of a *Tetrahymena* L-19 IVS RNA is sometimes utilized in which the nucleotide sequence of the active site is complementary to the nucleotide sequence to be cleaved in a *BRAF*-encoding mRNA. See, *e.g.*, Cech *et al* U.S. Patent No. 4,987,071; and Cech *et al* U.S. Patent No. 5,116,742. Also, *BRAF* mRNA can be used to select a catalytic RNA having a specific ribonuclease activity from a pool of RNA molecules. See, *e.g.*, Bartel & Szostak, *Science 261:* 1411-1418 (1993).

[00124] *BRAF* gene expression can be inhibited by targeting nucleotide sequences complementary to the regulatory region of the *BRAF* (*e.g.*, *BRAF* promoter and/or enhancers) to form triple helical structures that prevent transcription of the *BRAF* gene in target cells. See, Helene, *Anticancer Drug* Des. 6(6): 569-84 (1991); Helene *et al., Ann. N.Y. Acad. Sci. 660:* 27-36 (1992); and Maher, *Bioassays 14(12):* 807-15 (1992). Potential sequences that can be targeted for triple helix

formation can be increased by creating a so-called "switchback" nucleic acid molecule. Switchback molecules are synthesized in an alternating 5'-3', 3'-5' manner, such that they base pair with first one strand of a duplex and then the other, eliminating the necessity for a sizeable stretch of either purines or pyrimidines to be present on one strand of a duplex.

[00125] Gene expression may be inhibited by the introduction of double-stranded RNA (dsRNA), which induces potent and specific gene silencing, a phenomenon called RNA interference or RNAi. See, *e.g.*, Fire *et al.*, US Patent Number 6,506,559; Tuschl *et al.* PCT International Publication No. WO 01/75164; Kay *et al.* PCT International Publication No. WO 03/010180A1; or Bosher JM, Labouesse, *Nat Cell Biol* 2000 Feb;2(2):E31-6. This process has been improved by decreasing the size of the double-stranded RNA to 20-24 base pairs (to create small-interfering RNAs or siRNAs) that "switched off" genes in mammalian cells without initiating an acute phase response, *i.e.*, a host defense mechanism that often results in cell death. *See, e.g.*, Caplen et al. *Proc Natl Acad Sci* U S A. 2001 Aug 14;98(17):9742-7 and Elbashir SM et al. *Methods* 2002 Feb;26(2):199-213.

[00126] There is increasing evidence of post-transcriptional gene silencing by RNA interference (RNAi) for inhibiting targeted expression in mammalian cells at the mRNA level, in human cells. There is additional evidence of effective methods for inhibiting the proliferation and migration of tumor cells in human patients, and for inhibiting metastatic cancer development. *See, e.g.*, U.S. Patent Application Number US2001000993183; Caplen NJ et al. *Proc Natl Acad Sci* U S A; and Abderrahmani A. et al. *Mol Cell Biol* 2001 Nov21(21):7256-67.

[00127] An "siRNA" or "RNAi" refers to a nucleic acid that forms a double stranded RNA and has the ability to reduce or inhibit expression of a gene or target gene when the siRNA is delivered to or expressed in the same cell as the gene or target gene. "siRNA" thus refers to short double stranded RNA formed by the complementary strands. Complementary portions of the siRNA that hybridize to form the double stranded molecule often have substantial or complete identity to the target molecule sequence. In one embodiment, an siRNA refers to a nucleic acid that has substantial or complete identity to a target gene and forms a double stranded siRNA, such as a nucleotide sequence set forth in SEQ ID Nos: 1, 3A, 3B or 3C, for example.

[00128] When designing the siRNA molecules, the targeted region often is selected from a given DNA sequence beginning 50 to 100 nt downstream of the start codon. *See, e.g.*, Elbashir *et al,. Methods* 26:199-213 (2002). Initially, 5' or 3' UTRs and regions nearby the start codon were avoided assuming that UTR-binding proteins and/or translation initiation complexes may interfere with binding of the siRNP or RISC endonuclease complex. Sometimes regions of the target 23 nucleotides in length conforming to the sequence motif AA(N19)TT (N, an nucleotide), and regions with approximately 30% to 70% G/C-content (often about 50% G/C-content) often are selected. If no suitable sequences are found, the search often is extended using the motif NA(N21). The sequence of the sense siRNA sometimes corresponds to (N19) TT or N21 (position 3 to 23 of the 23-nt motif),

respectively. In the latter case, the 3' end of the sense siRNA often is converted to TT. The rationale for this sequence conversion is to generate a symmetric duplex with respect to the sequence composition of the sense and antisense 3' overhangs. The antisense siRNA is synthesized as the complement to position 1 to 21 of the 23-nt motif. Because position 1 of the 23-nt motif is not recognized sequence-specifically by the antisense siRNA, the 3'-most nucleotide residue of the antisense siRNA can be chosen deliberately. However, the penultimate nucleotide of the antisense siRNA (complementary to position 2 of the 23-nt motif) often is complementary to the targeted sequence. For simplifying chemical synthesis, TT often is utilized. siRNAs corresponding to the target motif NAR(N17)YNN, where R is purine (A,G) and Y is pyrimidine (C,U), often are selected. Respective 21 nucleotide sense and antisense siRNAs often begin with a purine nucleotide and can also be expressed from pol III expression vectors without a change in targeting site. Expression of RNAs from pol III promoters often is efficient when the first transcribed nucleotide is a purine.

[00129] The sequence of the siRNA can correspond to the full length target gene, or a subsequence thereof. Often, the siRNA is about 15 to about 50 nucleotides in length (*e.g.*, each complementary sequence of the double stranded siRNA is 15-50 nucleotides in length, and the double stranded siRNA is about 15-50 base pairs in length, somtimes about 20-30 nucleotides in length or about 20-25 nucleotides in length, *e.g.*, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, or 30 nucleotides in length. The siRNA often is about 21 nucleotides in length. Methods of using siRNA are well known in the art, and specific siRNA molecules may be purchased from a number of companies including Dharmacon Research, Inc.

[00130] Antisense, ribozyme, and modified *BRAF* nucleic acid molecules can be altered at base moieties, sugar moieties or phosphate backbone moieties to improve stability, hybridization, or solubility of the molecule. For example, the deoxyribose phosphate backbone of nucleic acid molecules can be modified to generate peptide nucleic acids (see Hyrup *et al., Bioorganic & Medicinal Chemistry 4 (1):* 5-23 (1996)). As used herein, the terms "peptide nucleic acid" or "PNA" refers to a nucleic acid mimic such as a DNA mimic, in which the deoxyribose phosphate backbone is replaced by a pseudopeptide backbone and only the four natural nucleobases are retained. The neutral backbone of a PNA can allow for specific hybridization to DNA and RNA under conditions of low ionic strength. Synthesis of PNA oligomers can be performed using standard solid phase peptide synthesis protocols as described, for example, in Hyrup *et al.*, (1996) *supra* and Perry-O'Keefe *et al., Proc. Natl. Acad. Sci. 93:* 14670-675 (1996).

[00131] PNAs of *BRAF* nucleic acids can be used in prognostic, diagnostic, and therapeutic applications. For example, PNAs can be used as antisense or antigene agents for sequence-specific modulation of gene expression by, for example, inducing transcription or translation arrest or inhibiting replication. PNAs of *BRAF* nucleic acid molecules can also be used in the analysis of single base pair mutations in a gene, (*e.g.*, by PNA-directed PCR clamping); as "artificial restriction

enzymes" when used in combination with other enzymes, (*e.g.*, S1 nucleases (Hyrup (1996) *supra*)); or as probes or primers for DNA sequencing or hybridization (Hyrup *et al.*, (1996) *supra*; Perry-O'Keefe *supra*).

[00132] In other embodiments, oligonucleotides may include other appended groups such as peptides (*e.g.*, for targeting host cell receptors *in vivo*), or agents facilitating transport across cell membranes (see, *e.g.*, Letsinger *et al., Proc. Natl. Acad. Sci. USA 86:* 6553-6556 (1989); Lemaitre *et al., Proc. Natl. Acad. Sci. USA 84:* 648-652 (1987); PCT Publication No. W088/09810) or the blood-brain barrier (see, *e.g.*, PCT Publication No. W089/10134). In addition, oligonucleotides can be modified with hybridization-triggered cleavage agents (See, *e.g.*, Krol *et al., Bio-Techniques 6:* 958-976 (1988)) or intercalating agents. (See, *e.g.*, Zon, *Pharm. Res. 5:* 539-549 (1988)). To this end, the oligonucleotide may be conjugated to another molecule, (*e.g.*, a peptide, hybridization triggered cross-linking agent, transport agent, or hybridization-triggered cleavage agent).

[00133] Also included herein are molecular beacon oligonucleotide primer and probe molecules having one or more regions which are complementary to a *BRAF* nucleic acid, two complementary regions one having a fluorophore and one a quencher such that the molecular beacon is useful for quantifying the presence of the *BRAF* nucleic acid in a sample. Molecular beacon nucleic acids are described, for example, in Lizardi *et al.*, U.S. Patent No. 5,854,033; Nazarenko *et al.*, U.S. Patent No. 5,866,336, and Livak *et al.*, U.S. Patent 5,876,930.

Anti-*BRAF* Antibodies

[00134] In an embodiment, antibodies are screened as test molecules and used as therapeutics for treating melanoma in a subject. The term "antibody" as used herein refers to an immunoglobulin molecule or immunologically active portion thereof, *i.e.*, an antigen-binding portion. Examples of immunologically active portions of immunoglobulin molecules include F(ab) and $F(ab')_2$ fragments which can be generated by treating the antibody with an enzyme such as pepsin. An antibody can be a polyclonal, monoclonal, recombinant, *e.g.*, a chimeric or humanized, fully human, non-human, *e.g.*, murine, or single chain antibody. An antibody may have effector function and can fix complement, and is sometimes coupled to a toxin or imaging agent.

[00135] A full-length *BRAF* polypeptide or, antigenic peptide fragment of *BRAF* can be used as an immunogen or can be used to identify anti-*BRAF* antibodies made with other immunogens, *e.g.*, cells, membrane preparations, and the like. The antigenic peptide of *BRAF* should include at least 8 amino acid residues of the amino acid sequences set forth in Figures 2A-2G and encompass an epitope of *BRAF*. Antigenic peptides include 10 or more amino acids, 15 or more amino acids, often 20 or more amino acids, and typically 30 or more amino acids. Hydrophilic and hydrophobic fragments of *BRAF* polypeptides can be used as immunogens.

[00136] Epitopes encompassed by the antigenic peptide are regions of *BRAF* located on the surface of the polypeptide (*e.g.*, hydrophilic regions) as well as regions with high antigenicity. For example, an Emini surface probability analysis of the human *BRAF* polypeptide sequence can be used to indicate the regions that have a particularly high probability of being localized to the surface of the *BRAF* polypeptide and are thus likely to constitute surface residues useful for targeting antibody production. The antibody may bind an epitope on any domain or region on *BRAF* polypeptides described herein.

[00137] Also, chimeric, humanized, and completely human antibodies are useful for applications which include repeated administration to subjects. Chimeric and humanized monoclonal antibodies, comprising both human and non-human portions, can be made using standard recombinant DNA techniques. Such chimeric and humanized monoclonal antibodies can be produced by recombinant DNA techniques known in the art, for example using methods described in Robinson *et al* International Application No. PCT/US86/02269; Akira, *et al* European Patent Application 184,187; Taniguchi, M., European Patent Application 171,496; Morrison *et al* European Patent Application 173,494; Neuberger *et al* PCT International Publication No. WO 86/01533; Cabilly *et al* U.S. Patent No. 4,816,567; Cabilly *et al* European Patent Application 125,023; Better *et al., Science 240:* 1041-1043 (1988); Liu *et al., Proc. Natl. Acad. Sci. USA 84:* 3439-3443 (1987); Liu *et al., J. Immunol. 139:* 3521-3526 (1987); Sun *et al., Proc. Natl. Acad. Sci. USA 84:* 214-218 (1987); Nishimura *et al., Canc. Res. 47:* 999-1005 (1987); Wood *et al., Nature 314:* 446-449 (1985); and Shaw *et al., J. Natl. Cancer Inst. 80:* 1553-1559 (1988); Morrison, S. L., *Science 229:* 1202-1207 (1985); Oi *et al., BioTechniques 4:* 214 (1986); Winter U.S. Patent 5,225,539; Jones *et al., Nature 321:* 552-525 (1986); Verhoeyan *et al., Science 239:* 1534; and Beidler *et al., J. Immunol. 141:* 4053-4060 (1988).

[00138] Completely human antibodies are particularly desirable for therapeutic treatment of human patients. Such antibodies can be produced using transgenic mice that are incapable of expressing endogenous immunoglobulin heavy and light chains genes, but which can express human heavy and light chain genes. *See*, for example, Lonberg and Huszar, *Int. Rev. Immunol. 13*: 65-93 (1995); and U.S. Patent Nos. 5,625,126; 5,633,425; 5,569,825; 5,661,016; and 5,545,806. In addition, companies such as Abgenix, Inc. (Fremont, CA) and Medarex, Inc. (Princeton, NJ), can be engaged to provide human antibodies directed against a selected antigen using technology similar to that described above. Completely human antibodies that recognize a selected epitope also can be generated using a technique referred to as "guided selection." In this approach a selected non-human monoclonal antibody (*e.g.*, a murine antibody) is used to guide the selection of a completely human antibody recognizing the same epitope. This technology is described for example by Jespers *et al., Bio/Technology 12:* 899-903 (1994).

[00139] An anti-*BRAF* antibody can be a single chain antibody. A single chain antibody (scFV) can be engineered (see, *e.g.*, Colcher *et al., Ann. N Y Acad. Sci. 880:* 263-80 (1999); and Reiter, *Clin.*

Cancer Res. 2: 245-52 (1996)). Single chain antibodies can be dimerized or multimerized to generate multivalent antibodies having specificities for different epitopes of the same target *BRAF* polypeptide.

[00140] Antibodies also may be selected or modified so that they exhibit reduced or no ability to bind an Fc receptor. For example, an antibody may be an isotype or subtype, fragment or other mutant, which does not support binding to an Fc receptor (*e.g.*, it has a mutagenized or deleted Fc receptor binding region).

[00141] Also, an antibody (or fragment thereof) may be conjugated to a therapeutic moiety such as a cytotoxin, a therapeutic agent or a radioactive metal ion. A cytotoxin or cytotoxic agent includes any agent that is detrimental to cells. Examples include taxol, cytochalasin B, gramicidin D, ethidium bromide, emetine, mitomycin, etoposide, tenoposide, vincristine, vinblastine, colchicin, doxorubicin, daunorubicin, dihydroxy anthracin dione, mitoxantrone, mithramycin, actinomycin D, 1-dehydrotestosterone, glucocorticoids, procaine, tetracaine, lidocaine, propranolol, and puromycin and analogs or homologs thereof. Therapeutic agents include, but are not limited to, antimetabolites (*e.g.*, methotrexate, 6-mercaptopurine, 6-thioguanine, cytarabine, 5-fluorouracil decarbazine), alkylating agents (*e.g.*, mechlorethamine, thiotepa chlorambucil, melphalan, carmustine (BCNU) and lomustine (CCNU), cyclophosphamide, busulfan, dibromomannitol, streptozotocin, mitomycin C, and cis-dichlorodiamine platinum (II) (DDP) cisplatin), anthracyclines (*e.g.*, daunorubicin (formerly daunomycin) and doxorubicin), antibiotics (*e.g.*, dactinomycin (formerly actinomycin), bleomycin, mithramycin, and anthramycin (AMC)), and anti-mitotic agents (*e.g.*, vincristine and vinblastine).

[00142] Antibody conjugates can be used for modifying a given biological response. For example, the drug moiety may be a protein or polypeptide possessing a desired biological activity. Such proteins may include, for example, a toxin such as abrin, ricin A, pseudomonas exotoxin, or diphtheria toxin; a polypeptide such as tumor necrosis factor, □-interferon, □-interferon, nerve growth factor, platelet derived growth factor, tissue plasminogen activator; or, biological response modifiers such as, for example, lymphokines, interleukin-1 ("IL-1"), interleukin-2 ("IL-2"), interleukin-6 ("IL-6"), granulocyte macrophage colony stimulating factor ("GM-CSF"), granulocyte colony stimulating factor ("G-CSF"), or other growth factors. Also, an antibody can be conjugated to a second antibody to form an antibody heteroconjugate as described by Segal in U.S. Patent No. 4,676,980, for example.

[00143] An anti-*BRAF* antibody (*e.g.*, monoclonal antibody) can be used to isolate *BRAF* polypeptides by standard techniques, such as affinity chromatography or immunoprecipitation. Moreover, an anti-*BRAF* antibody can be used to detect a *BRAF* polypeptide (*e.g.*, in a cellular lysate or cell supernatant) in order to evaluate the abundance and pattern of expression of the polypeptide. Anti-*BRAF* antibodies can be used diagnostically to monitor polypeptide levels in tissue as part of a clinical testing procedure, *e.g.*, to determine the efficacy of a given treatment regimen. Detection can be facilitated by coupling (*i.e.*, physically linking) the antibody to a detectable substance (*i.e.*,

antibody labeling). Examples of detectable substances include various enzymes, prosthetic groups, fluorescent materials, luminescent materials, bioluminescent materials, and radioactive materials. Examples of suitable enzymes include horseradish peroxidase, alkaline phosphatase, □-galactosidase, or acetylcholinesterase; examples of suitable prosthetic group complexes include streptavidin/biotin and avidin/biotin; examples of suitable fluorescent materials include umbelliferone, fluorescein, fluorescein isothiocyanate, rhodamine, dichlorotriazinylamine fluorescein, dansyl chloride or phycoerythrin; an example of a luminescent material includes luminol; examples of bioluminescent materials include luciferase, luciferin, and aequorin, and examples of suitable radioactive material include ^{125}I, ^{131}I, ^{35}S or ^{3}H. Also, an anti-*BRAF* antibody can be utilized as a test molecule for determining whether it can treat melanoma, and as a therapeutic for administration to a subject for treating melanoma.

[00144] An antibody can be made by immunizing with a purified *BRAF* antigen, or a fragment thereof, *e.g.*, a fragment described herein, a membrane associated antigen, tissues, *e.g.*, crude tissue preparations, whole cells, preferably living cells, lysed cells, or cell fractions.

[00145] Included herein are antibodies which bind only a native *BRAF* polypeptide, only denatured or otherwise non-native *BRAF* polypeptide, or which bind both, as well as those having linear or conformational epitopes. Conformational epitopes sometimes can be identified by selecting antibodies that bind to native but not denatured *BRAF* polypeptide.

Screening Assays

[00146] As used herein, the term "system" refers to a cell free *in vitro* environment and a cell-based environment such as a collection of cells, a tissue, an organ, or an organism. A system is "contacted" with a test molecule in a variety of manners, including adding molecules in solution and allowing them to interact with one another by diffusion, cell injection, and any administration routes in an animal. As used herein, the term "interaction" refers to an effect of a test molecule on a *BRAF* nucleic acid, polypeptide, or variant thereof (collectively referred to as a "*BRAF* molecule"), where the effect is sometimes binding between the test molecule and the nucleic acid or polypeptide, and is often an observable change in cells, tissue, or organism.

[00147] There are many standard methods for detecting the presence or absence of interaction between a test molecule and a *BRAF* nucleic acid or polypeptide. For example, titrametric, acidimetric, radiometric, NMR, monolayer, polarographic, spectrophotometric, fluorescent, and ESR assays probative of *BRAF* function may be utilized.

[00148] *BRAF* activity and/or *BRAF* interactions can be detected and quantified using assays known in the art. For example, an immunoprecipitation assay or a kinase activity assay that employs a kinase-inactivated MEK can be utilized. Kinase inactivated MEKs are known in the art, such as a MEK that includes the mutation K97M. In these assays, mammalian cells (e.g., COS or NIH-3T3) are

transiently transfected with constructs expressing *BRAF*, and in addition, the cells are co-transfected with oncogenic *RAS* or *SRC* or both. Oncogenic *RAS* or *SRC* activates *BRAF* kinase activity. *BRAF* is immunoprecipitated from cell extracts using a monoclonal antibody (e.g., 9E10) or a polyclonal antibody (e.g., from rabbit) specific for a unique peptide from *BRAF*. *BRAF* is then resuspended in assay buffer containing GST-Mek1 or GST-Mek2 and/or GST-ERK2. In addition, [γ P^{32}] ATP can be added to detect and/or quantify phosphorylation activity. Samples are incubated for 5-30 minutes at 30°C, and then the reaction is terminated by addition of EDTA. The samples are centrifuged and the supernatant fractions are collected. Phosphorylation activity is detected using one of two methods: (i) activity of GST-ERK2 kinase can be measured using MBP (myelin basic protein, a substrate for ERK) as substrate, or (ii) following incubation of immunoprecipitated *BRAF* in reaction buffer containing GST-ERK and [γ P^{32}] ATP, transfer of labeled ATP to kinase-dead ERK can be quantified by a phosphor-imager or densitometer following PAGE separation of polypeptide products (phosphorylated and non-phosphorylated forms). Examples of assays are described in Weber *et al., Oncogene 19*: 169-176 (2000); Mason *et al., EMBO J. 18*: 2137-2148 (1999); Marais *et al., J. Biol. Chem. 272*: 4378-4383 (1997); Marais *et al., EMBO J. 14*: 3136-3145 (1995).

[00149] An interaction can be determined by labeling the test molecule and/or the *BRAF* molecule, where the label is covalently or non-covalently attached to the test molecule or *BRAF* molecule. The label is sometimes a radioactive molecule such as ^{125}I, ^{131}I, ^{35}S or ^{3}H, which can be detected by direct counting of radioemission or by scintillation counting. Also, enzymatic labels such as horseradish peroxidase, alkaline phosphatase, or luciferase may be utilized where the enzymatic label can be detected by determining conversion of an appropriate substrate to product. Also, presence or absence of an interaction can be determined without labeling. For example, a microphysiometer (*e.g.*, Cytosensor) is an analytical instrument that measures the rate at which a cell acidifies its environment using a light-addressable potentiometric sensor (LAPS). Changes in this acidification rate can be used as an indication of an interaction between a test molecule and *BRAF* (McConnell, H. M. *et al., Science 257:* 1906-1912 (1992)).

[00150] In cell-based systems, cells typically include a *BRAF* nucleic acid or polypeptide or variants thereof and are often of mammalian origin, although the cell can be of any origin. Whole cells, cell homogenates, and cell fractions (*e.g.*, cell membrane fractions) can be subjected to analysis. Where interactions between a test molecule with a *BRAF* polypeptide or variant thereof are monitored, soluble and/or membrane bound forms of the polypeptide or variant may be utilized. Where membrane-bound forms of the polypeptide are used, it may be desirable to utilize a solubilizing agent. Examples of such solubilizing agents include non-ionic detergents such as n-octylglucoside, n-dodecylglucoside, n-dodecylmaltoside, octanoyl-N-methylglucamide, decanoyl-N-methylglucamide, Triton® X-100, Triton® X-114, Thesit®, Isotridecypoly(ethylene glycol ether)$_n$, 3-[(3-cholamidopropyl)dimethylamminio]-1-propane sulfonate (CHAPS), 3-[(3-

cholamidopropyl)dimethylamminio]-2-hydroxy-1-propane sulfonate (CHAPSO), or N-dodecyl-N,N-dimethyl-3-ammonio-1-propane sulfonate.

[00151] An interaction between two molecules can also be detected by monitoring fluorescence energy transfer (FET) (see, for example, Lakowicz *et al.*, U.S. Patent No. 5,631,169; Stavrianopoulos *et al.* U.S. Patent No. 4,868,103). A fluorophore label on a first, "donor" molecule is selected such that its emitted fluorescent energy will be absorbed by a fluorescent label on a second, "acceptor" molecule, which in turn is able to fluoresce due to the absorbed energy. Alternately, the "donor" polypeptide molecule may simply utilize the natural fluorescent energy of tryptophan residues. Labels are chosen that emit different wavelengths of light, such that the "acceptor" molecule label may be differentiated from that of the "donor". Since the efficiency of energy transfer between the labels is related to the distance separating the molecules, the spatial relationship between the molecules can be assessed. In a situation in which binding occurs between the molecules, the fluorescent emission of the "acceptor" molecule label in the assay should be maximal. An FET binding event can be conveniently measured through standard fluorometric detection means well known in the art (*e.g.*, using a fluorimeter).

[00152] In another embodiment, determining the presence or absence of an interaction between a test molecule and a *BRAF* molecule can be effected by using real-time Biomolecular Interaction Analysis (BIA) (see, *e.g.*, Sjolander & Urbaniczk, *Anal. Chem. 63:* 2338-2345 (1991) and Szabo *et al., Curr. Opin. Struct. Biol. 5:* 699-705 (1995)). "Surface plasmon resonance" or "BIA" detects biospecific interactions in real time, without labeling any of the interactants (*e.g.*, BIAcore). Changes in the mass at the binding surface (indicative of a binding event) result in alterations of the refractive index of light near the surface (the optical phenomenon of surface plasmon resonance (SPR)), resulting in a detectable signal which can be used as an indication of real-time reactions between biological molecules.

[00153] In another embodiment, the *BRAF* molecule or test molecules are anchored to a solid phase. The *BRAF* molecule/test molecule complexes anchored to the solid phase can be detected at the end of the reaction. The target *BRAF* molecule is often anchored to a solid surface, and the test molecule, which is not anchored, can be labeled, either directly or indirectly, with detectable labels discussed herein.

[00154] It may be desirable to immobilize a *BRAF* molecule, an anti-*BRAF* antibody, or test molecules to facilitate separation of complexed from uncomplexed forms of *BRAF* molecules and test molecules, as well as to accommodate automation of the assay. Binding of a test molecule to a *BRAF* molecule can be accomplished in any vessel suitable for containing the reactants. Examples of such vessels include microtitre plates, test tubes, and micro-centrifuge tubes. In one embodiment, a fusion polypeptide can be provided which adds a domain that allows a *BRAF* molecule to be bound to a matrix. For example, glutathione-S-transferase/*BRAF* fusion polypeptides or glutathione-S-

transferase/target fusion polypeptides can be adsorbed onto glutathione sepharose beads (Sigma Chemical, St. Louis, MO) or glutathione derivatized microtitre plates, which are then combined with the test compound or the test compound and either the non-adsorbed target polypeptide or *BRAF* polypeptide, and the mixture incubated under conditions conducive to complex formation (*e.g.*, at physiological conditions for salt and pH). Following incubation, the beads or microtitre plate wells are washed to remove any unbound components, the matrix immobilized in the case of beads, complex determined either directly or indirectly, for example, as described above. Alternatively, the complexes can be dissociated from the matrix, and the level of *BRAF* binding or activity determined using standard techniques.

[00155] Other techniques for immobilizing a *BRAF* molecule on matrices include using biotin and streptavidin. For example, biotinylated *BRAF* polypeptide or target molecules can be prepared from biotin-NHS (N-hydroxy-succinimide) using techniques known in the art (*e.g.*, biotinylation kit, Pierce Chemicals, Rockford, IL), and immobilized in the wells of streptavidin-coated 96 well plates (Pierce Chemical).

[00156] In order to conduct the assay, the non-immobilized component is added to the coated surface containing the anchored component. After the reaction is complete, unreacted components are removed (*e.g.*, by washing) under conditions such that any complexes formed will remain immobilized on the solid surface. The detection of complexes anchored on the solid surface can be accomplished in a number of ways. Where the previously non-immobilized component is pre-labeled, the detection of label immobilized on the surface indicates that complexes were formed. Where the previously non-immobilized component is not pre-labeled, an indirect label can be used to detect complexes anchored on the surface; *e.g.*, using a labeled antibody specific for the immobilized component (the antibody, in turn, can be directly labeled or indirectly labeled with, *e.g.*, a labeled anti-Ig antibody).

[00157] In one embodiment, this assay is performed utilizing antibodies reactive with *BRAF* polypeptide or test molecules but which do not interfere with binding of the *BRAF* polypeptide to its test molecule. Such antibodies can be derivatized to the wells of the plate, and unbound target or *BRAF* polypeptide trapped in the wells by antibody conjugation. Methods for detecting such complexes, in addition to those described above for the GST-immobilized complexes, include immunodetection of complexes using antibodies reactive with the *BRAF* polypeptide or target molecule, as well as enzyme-linked assays which rely on detecting an enzymatic activity associated with the *BRAF* polypeptide or test molecule.

[00158] Alternatively, cell free assays can be conducted in a liquid phase. In such an assay, the reaction products are separated from unreacted components, by any of a number of standard techniques, including but not limited to: differential centrifugation (see, for example, Rivas, G., and Minton, A. P., *Trends Biochem Sci Aug;18(8):* 284-7 (1993)); chromatography (gel filtration

chromatography, ion-exchange chromatography); electrophoresis (see, *e.g.*, Ausubel *et al., eds. Current Protocols in Molecular Biology, J. Wiley:* New York (1999)); and immunoprecipitation (see, for example, Ausubel, F. *et al., eds. Current Protocols in Molecular Biology, J. Wiley:* New York (1999)). Such resins and chromatographic techniques are known to one skilled in the art (*see, e.g.*, Heegaard, *J Mol. Recognit. Winter; 11(1-6):* 141-8 (1998); Hage & Tweed, *J. Chromatogr. B Biomed. Sci. Appl. Oct 10; 699 (1-2): 499-525* (1997)). Further, fluorescence energy transfer may also be conveniently utilized, as described herein, to detect binding without further purification of the complex from solution.

[00159] In another embodiment, modulators of *BRAF* expression are identified. For example, a cell or cell free mixture is contacted with a candidate compound and the expression of *BRAF* mRNA or polypeptide evaluated relative to the level of expression of *BRAF* mRNA or polypeptide in the absence of the candidate compound. When expression of *BRAF* mRNA or polypeptide is greater in the presence of the candidate compound than in its absence, the candidate compound is identified as a stimulator of *BRAF* mRNA or polypeptide expression. Alternatively, when expression of *BRAF* mRNA or polypeptide is less (statistically significantly less) in the presence of the candidate compound than in its absence, the candidate compound is identified as an inhibitor of *BRAF* mRNA or polypeptide expression. The level of *BRAF* mRNA or polypeptide expression can be determined by methods described herein for detecting *BRAF* mRNA or polypeptide.

BRAF Binding Partners

[00160] In another embodiment, binding partners that interact with a *BRAF* molecule are detected. The *BRAF* molecules can interact with one or more cellular or extracellular macromolecules, such as polypeptides, in vivo, and these molecules that interact with *BRAF* molecules are referred to herein as "binding partners." Molecules that disrupt such interactions can be useful in regulating the activity of the target gene product. Such molecules can include, but are not limited to molecules such as antibodies, peptides, and small molecules (*e.g.*, siRNA). The preferred target genes/products for use in this embodiment are the *BRAF* genes herein identified. In an alternative embodiment, provided are methods for determining the ability of the test compound to modulate the activity of a *BRAF* polypeptide through modulation of the activity of a downstream effector of a *BRAF* target molecule. For example, the activity of the effector molecule on an appropriate target can be determined, or the binding of the effector to an appropriate target can be determined, as previously described.

[00161] To identify compounds that interfere with the interaction between the target gene product and its cellular or extracellular binding partner(s), *e.g.*, a substrate, a reaction mixture containing the target gene product and the binding partner is prepared, under conditions and for a time sufficient, to allow the two products to form complex. In order to test an inhibitory agent, the reaction

mixture is provided in the presence and absence of the test compound. The test compound can be initially included in the reaction mixture, or can be added at a time subsequent to the addition of the target gene and its cellular or extracellular binding partner. Control reaction mixtures are incubated without the test compound or with a placebo. The formation of any complexes between the target gene product and the cellular or extracellular binding partner is then detected. The formation of a complex in the control reaction, but not in the reaction mixture containing the test compound, indicates that the compound interferes with the interaction of the target gene product and the interactive binding partner. Additionally, complex formation within reaction mixtures containing the test compound and normal target gene product can also be compared to complex formation within reaction mixtures containing the test compound and mutant target gene product. This comparison can be important in those cases wherein it is desirable to identify compounds that disrupt interactions of mutant but not normal target gene products.

[00162] These assays can be conducted in a heterogeneous or homogeneous format. Heterogeneous assays involve anchoring either the target gene product or the binding partner onto a solid phase, and detecting complexes anchored on the solid phase at the end of the reaction. In homogeneous assays, the entire reaction is carried out in a liquid phase. In either approach, the order of addition of reactants can be varied to obtain different information about the compounds being tested. For example, test compounds that interfere with the interaction between the target gene products and the binding partners, *e.g.*, by competition, can be identified by conducting the reaction in the presence of the test substance. Alternatively, test compounds that disrupt preformed complexes, *e.g.*, compounds with higher binding constants that displace one of the components from the complex, can be tested by adding the test compound to the reaction mixture after complexes have been formed. The various formats are briefly described below.

[00163] In a heterogeneous assay system, either the target gene product or the interactive cellular or extracellular binding partner, is anchored onto a solid surface (*e.g.*, a microtitre plate), while the non-anchored species is labeled, either directly or indirectly. The anchored species can be immobilized by non-covalent or covalent attachments. Alternatively, an immobilized antibody specific for the species to be anchored can be used to anchor the species to the solid surface.

[00164] In order to conduct the assay, the partner of the immobilized species is exposed to the coated surface with or without the test compound. After the reaction is complete, unreacted components are removed (*e.g.*, by washing) and any complexes formed will remain immobilized on the solid surface. Where the non-immobilized species is pre-labeled, the detection of label immobilized on the surface indicates that complexes were formed. Where the non-immobilized species is not pre-labeled, an indirect label can be used to detect complexes anchored on the surface; *e.g.*, using a labeled antibody specific for the initially non-immobilized species (the antibody, in turn, can be directly labeled or indirectly labeled with, *e.g.*, a labeled anti-Ig antibody). Depending upon

the order of addition of reaction components, test compounds that inhibit complex formation or that disrupt preformed complexes can be detected.

[00165] Alternatively, the reaction can be conducted in a liquid phase in the presence or absence of the test compound, the reaction products separated from unreacted components, and complexes detected; *e.g.*, using an immobilized antibody specific for one of the binding components to anchor any complexes formed in solution, and a labeled antibody specific for the other partner to detect anchored complexes. Again, depending upon the order of addition of reactants to the liquid phase, test compounds that inhibit complex or that disrupt preformed complexes can be identified.

[00166] In an alternate embodiment, a homogeneous assay can be used. For example, a preformed complex of the target gene product and the interactive cellular or extracellular binding partner product is prepared in that either the target gene products or their binding partners are labeled, but the signal generated by the label is quenched due to complex formation (see, *e.g.*, U.S. Patent No. 4,109,496 that utilizes this approach for immunoassays). The addition of a test substance that competes with and displaces one of the species from the preformed complex will result in the generation of a signal above background. In this way, test substances that disrupt target gene product-binding partner interaction can be identified.

[00167] Also, binding partners of *BRAF* molecules can be identified in a two-hybrid assay or three-hybrid assay (see, *e.g.*, U.S. Patent No. 5,283,317; Zervos *et al., Cell 72:223-232* (1993); Madura *et al., J. Biol. Chem. 268:* 12046-12054 (1993); Bartel *et al., Biotechniques 14:* 920-924 (1993); Iwabuchi *et al., Oncogene 8:* 1693-1696 (1993); and Brent WO94/10300), to identify other polypeptides, which bind to or interact with *BRAF* ("*BRAF*-binding polypeptides" or "*BRAF*-bp") and are involved in *BRAF* activity. Such *BRAF*-bps can be activators or inhibitors of signals by the *BRAF* polypeptides or *BRAF* targets as, for example, downstream elements of a *BRAF*-mediated signaling pathway.

[00168] A two-hybrid system is based on the modular nature of most transcription factors, which consist of separable DNA-binding and activation domains. Briefly, the assay utilizes two different DNA constructs. In one construct, the gene that codes for a *BRAF* polypeptide is fused to a gene encoding the DNA binding domain of a known transcription factor (*e.g.*, GAL-4). In the other construct, a DNA sequence, from a library of DNA sequences, that encodes an unidentified polypeptide ("prey" or "sample") is fused to a gene that codes for the activation domain of the known transcription factor. (Alternatively the: *BRAF* polypeptide can be the fused to the activator domain.) If the "bait" and the "prey" polypeptides are able to interact, *in vivo,* forming a *BRAF*-dependent complex, the DNA-binding and activation domains of the transcription factor are brought into close proximity. This proximity allows transcription of a reporter gene (*e.g.*, LacZ) which is operably linked to a transcriptional regulatory site responsive to the transcription factor. Expression of the reporter gene can be detected and cell colonies containing the functional transcription factor can be

isolated and used to obtain the cloned gene which encodes the polypeptide which interacts with the *BRAF* polypeptide.

Identification of Candidate Therapeutics

[00169] Candidate therapeutics for treating melanoma are identified from a group of test molecules that interact with a BRAF nucleic acid or polypeptide. Test molecules are normally ranked according to the degree with which they interact or modulate (*e.g.*, agonize or antagonize) DNA replication and/or processing, RNA transcription and/or processing, polypeptide production and/or processing, and/or function of *BRAF* molecules, for example, and then top ranking modulators are selected. Also, pharmacogenomic information described herein can determine the rank of a modulator. Candidate therapeutics typically are formulated for administration to a subject.

Therapeutic Treatments

[00170] Formulations or pharmaceutical compositions typically include in combination with a pharmaceutically acceptable carrier a compound, an antisense nucleic acid, a ribozyme, an antibody, a binding partner that interacts with a *BRAF* polypeptide, a *BRAF* nucleic acid, or a fragment thereof. The formulated molecule may be one that is identified by a screening method described above. Also, formulations may comprise a *BRAF* polypeptide or fragment thereof and a pharmaceutically acceptable carrier. As used herein, the term "pharmaceutically acceptable carrier" includes solvents, dispersion media, coatings, antibacterial and antifungal agents, isotonic and absorption delaying agents, and the like, compatible with pharmaceutical administration. Supplementary active compounds can also be incorporated into the compositions.

[00171] A pharmaceutical composition is formulated to be compatible with its intended route of administration. Examples of routes of administration include parenteral, *e.g.*, intravenous, intradermal, subcutaneous, oral (*e.g.*, inhalation), transdermal (topical), transmucosal, and rectal administration. Solutions or suspensions used for parenteral, intradermal, or subcutaneous application can include the following components: a sterile diluent such as water for injection, saline solution, fixed oils, polyethylene glycols, glycerin, propylene glycol or other synthetic solvents; antibacterial agents such as benzyl alcohol or methyl parabens; antioxidants such as ascorbic acid or sodium bisulfite; chelating agents such as ethylenediaminetetraacetic acid; buffers such as acetates, citrates or phosphates and agents for the adjustment of tonicity such as sodium chloride or dextrose. pH can be adjusted with acids or bases, such as hydrochloric acid or sodium hydroxide. The parenteral preparation can be enclosed in ampoules, disposable syringes or multiple dose vials made of glass or plastic.

[00172] Oral compositions generally include an inert diluent or an edible carrier. For the purpose of oral therapeutic administration, the active compound can be incorporated with excipients and used in the form of tablets, troches, or capsules, *e.g.*, gelatin capsules. Oral compositions can also

be prepared using a fluid carrier for use as a mouthwash. Pharmaceutically compatible binding agents, and/or adjuvant materials can be included as part of the composition. The tablets, pills, capsules, troches and the like can contain any of the following ingredients, or compounds of a similar nature: a binder such as microcrystalline cellulose, gum tragacanth or gelatin; an excipient such as starch or lactose, a disintegrating agent such as alginic acid, Primogel, or corn starch; a lubricant such as magnesium stearate or Sterotes; a glidant such as colloidal silicon dioxide; a sweetening agent such as sucrose or saccharin; or a flavoring agent such as peppermint, methyl salicylate, or orange flavoring.

[00173] Pharmaceutical compositions suitable for injectable use include sterile aqueous solutions (where water soluble) or dispersions and sterile powders for the extemporaneous preparation of sterile injectable solutions or dispersion. For intravenous administration, suitable carriers include physiological saline, bacteriostatic water, Cremophor EL™ (BASF, Parsippany, NJ) or phosphate buffered saline (PBS). In all cases, the composition must be sterile and should be fluid to the extent that easy syringability exists. It should be stable under the conditions of manufacture and storage and must be preserved against the contaminating action of microorganisms such as bacteria and fungi. The carrier can be a solvent or dispersion medium containing, for example, water, ethanol, polyol (for example, glycerol, propylene glycol, and liquid polyethylene glycol, and the like), and suitable mixtures thereof. The proper fluidity can be maintained, for example, by the use of a coating such as lecithin, by the maintenance of the required particle size in the case of dispersion and by the use of surfactants. Prevention of the action of microorganisms can be achieved by various antibacterial and antifungal agents, for example, parabens, chlorobutanol, phenol, ascorbic acid, thimerosal, and the like. In many cases, it will be preferable to include isotonic agents, for example, sugars, polyalcohols such as mannitol, sorbitol, sodium chloride in the composition. Prolonged absorption of the injectable compositions can be brought about by including in the composition an agent which delays absorption, for example, aluminum monostearate and gelatin.

[00174] Sterile injectable solutions can be prepared by incorporating the active compound in the required amount in an appropriate solvent with one or a combination of ingredients enumerated above, as required, followed by filtered sterilization. Generally, dispersions are prepared by incorporating the active compound into a sterile vehicle which contains a basic dispersion medium and the required other ingredients from those enumerated above. In the case of sterile powders for the preparation of sterile injectable solutions, the methods of preparation often utilized are vacuum drying and freeze-drying which yields a powder of the active ingredient plus any additional desired ingredient from a previously sterile-filtered solution thereof.

[00175] For administration by inhalation, the compounds are delivered in the form of an aerosol spray from pressured container or dispenser which contains a suitable propellant, *e.g.*, a gas such as carbon dioxide, or a nebulizer.

[00176] Systemic administration can also be by transmucosal or transdermal means. For transmucosal or transdermal administration, penetrants appropriate to the barrier to be permeated are used in the formulation. Such penetrants are generally known in the art, and include, for example, for transmucosal administration, detergents, bile salts, and fusidic acid derivatives. Transmucosal administration can be accomplished through the use of nasal sprays or suppositories. For transdermal administration, the active compounds are formulated into ointments, salves, gels, or creams (*e.g.*, sunscreen) as generally known in the art. Molecules can also be prepared in the form of suppositories (*e.g.*, with conventional suppository bases such as cocoa butter and other glycerides) or retention enemas for rectal delivery.

[00177] In one embodiment, active molecules are prepared with carriers that will protect the compound against rapid elimination from the body, such as a controlled release formulation, including implants and microencapsulated delivery systems. Biodegradable, biocompatible polymers can be used, such as ethylene vinyl acetate, polyanhydrides, polyglycolic acid, collagen, polyorthoesters, and polylactic acid. Methods for preparation of such formulations will be apparent to those skilled in the art. Materials can also be obtained commercially from Alza Corporation and Nova Pharmaceuticals, Inc. Liposomal suspensions (including liposomes targeted to infected cells with monoclonal antibodies to viral antigens) can also be used as pharmaceutically acceptable carriers. These can be prepared according to methods known to those skilled in the art, for example, as described in U.S. Patent No. 4,522,811.

[00178] It is advantageous to formulate oral or parenteral compositions in dosage unit form for ease of administration and uniformity of dosage. Dosage unit form as used herein refers to physically discrete units suited as unitary dosages for the subject to be treated; each unit containing a predetermined quantity of active compound calculated to produce the desired therapeutic effect in association with the required pharmaceutical carrier.

[00179] Toxicity and therapeutic efficacy of such compounds can be determined by standard pharmaceutical procedures in cell cultures or experimental animals, *e.g.*, for determining the LD_{50} (the dose lethal to 50% of the population) and the ED_{50} (the dose therapeutically effective in 50% of the population). The dose ratio between toxic and therapeutic effects is the therapeutic index and it can be expressed as the ratio LD_{50}/ED_{50}. Molecules which exhibit high therapeutic indices often are utilized. While molecules that exhibit toxic side effects may be used, care should be taken to design a delivery system that targets such compounds to the site of affected tissue in order to minimize potential damage to uninfected cells and, thereby, reduce side effects.

[00180] The data obtained from the cell culture assays and animal studies can be used in formulating a range of dosage for use in humans. The dosage of such molecules lies preferably within a range of circulating concentrations that include the ED_{50} with little or no toxicity. The dosage may vary within this range depending upon the dosage form employed and the route of administration

utilized. For any molecules used in the method, the therapeutically effective dose can be estimated initially from cell culture assays. A dose may be formulated in animal models to achieve a circulating plasma concentration range that includes the IC_{50} (*i.e.*, the concentration of the test compound which achieves a half-maximal inhibition of symptoms) as determined in cell culture. Such information can be used to more accurately determine useful doses in humans. Levels in plasma may be measured, for example, by high performance liquid chromatography.

[00181] As defined herein, a therapeutically effective amount of protein or polypeptide (*i.e.*, an effective dosage) ranges from about 0.001 to 30 mg/kg body weight, sometimes about 0.01 to 25 mg/kg body weight, often about 0.1 to 20 mg/kg body weight, and more often about 1 to 10 mg/kg, 2 to 9 mg/kg, 3 to 8 mg/kg, 4 to 7 mg/kg, or 5 to 6 mg/kg body weight. The protein or polypeptide can be administered one time per week for between about 1 to 10 weeks, sometimes between 2 to 8 weeks, often between about 3 to 7 weeks, and more often for about 4, 5, or 6 weeks. The skilled artisan will appreciate that certain factors may influence the dosage and timing required to effectively treat a subject, including but not limited to the severity of the disease or disorder, previous treatments, the general health and/or age of the subject, and other diseases present. Moreover, treatment of a subject with a therapeutically effective amount of a protein, polypeptide, or antibody can include a single treatment or, preferably, can include a series of treatments.

[00182] With regard to polypeptide formulations, featured herein is a method for treating melanoma in a subject, which comprises contacting one or more cells in the subject with a first *BRAF* polypeptide, where genomic DNA in the subject comprises a second *BRAF* nucleic acid having one or more polymorphic variations associated with melanoma. The first *BRAF* polypeptide comprises fewer polymorphic variations associated with melanoma than the second *BRAF* polypeptide. The first and second *BRAF* polypeptides are encoded by a nucleic acid which comprises a nucleotide sequence selected from the group consisting of the nucleotide sequence of SEQ ID NO: 1; a nucleotide sequence which encodes a polypeptide consisting of an amino acid sequence set forth in Figures 2A-2G; and a nucleotide sequence which encodes a polypeptide that is 90% or more identical to an amino acid sequence set forth in Figures 2A-2G. The second *BRAF* polypeptide also may be encoded by a fragment of the foregoing nucleic acids comprising the one or more polymorphic variations. The subject is often a human.

[00183] For antibodies, a dosage of 0.1 mg/kg of body weight (generally 10 mg/kg to 20 mg/kg) is often utilized. If the antibody is to act in the brain, a dosage of 50 mg/kg to 100 mg/kg is often appropriate. Generally, partially human antibodies and fully human antibodies have a longer half-life within the human body than other antibodies. Accordingly, lower dosages and less frequent administration is often possible. Modifications such as lipidation can be used to stabilize antibodies and to enhance uptake and tissue penetration (*e.g.*, into the brain). A method for lipidation of

antibodies is described by Cruikshank *et al., J. Acquired Immune Deficiency Syndromes and Human Retrovirology 14:*193 (1997).

[00184] Antibody conjugates can be used for modifying a given biological response, the drug moiety is not to be construed as limited to classical chemical therapeutic agents. For example, the drug moiety may be a protein or polypeptide possessing a desired biological activity. Such proteins may include, for example, a toxin such as abrin, ricin A, pseudomonas exotoxin, or diphtheria toxin; a polypeptide such as tumor necrosis factor, .alpha.-interferon, .beta.-interferon, nerve growth factor, platelet derived growth factor, tissue plasminogen activator; or, biological response modifiers such as, for example, lymphokines, interleukin-1 ("IL-1"), interleukin-2 ("IL-2"), interleukin-6 ("IL-6"), granulocyte macrophage colony stimulating factor ("GM-CSF"), granulocyte colony stimulating factor ("G-CSF"), or other growth factors. Alternatively, an antibody can be conjugated to a second antibody to form an antibody heteroconjugate as described by Segal in U.S. Patent No. 4,676,980.

[00185] For compounds, exemplary doses include milligram or microgram amounts of the compound per kilogram of subject or sample weight, for example, about 1 microgram per kilogram to about 500 milligrams per kilogram, about 100 micrograms per kilogram to about 5 milligrams per kilogram, or about 1 microgram per kilogram to about 50 micrograms per kilogram. It is understood that appropriate doses of a small molecule depend upon the potency of the small molecule with respect to the expression or activity to be modulated. When one or more of these small molecules is to be administered to an animal (*e.g.*, a human) in order to modulate expression or activity of a polypeptide or nucleic acid, a physician, veterinarian, or researcher may, for example, prescribe a relatively low dose at first, subsequently increasing the dose until an appropriate response is obtained. In addition, it is understood that the specific dose level for any particular animal subject will depend upon a variety of factors including the activity of the specific compound employed, the age, body weight, general health, gender, and diet of the subject, the time of administration, the route of administration, the rate of excretion, any drug combination, and the degree of expression or activity to be modulated.

[00186] *BRAF* nucleic acid molecules can be inserted into vectors and used in gene therapy methods for treating melanoma. Featured herein is a method for treating melanoma in a subject, which comprises contacting one or more cells in the subject with a first *BRAF* nucleic acid. Genomic DNA in the subject comprises a second *BRAF* nucleic acid comprising one or more polymorphic variations associated with melanoma, and the first *BRAF* nucleic acid comprises fewer polymorphic variations associated with melanoma. The first and second *BRAF* nucleic acids typically comprise a nucleotide sequence selected from the group consisting of the nucleotide sequence of SEQ ID NO: 1; a nucleotide sequence which encodes a polypeptide consisting of an amino acid sequence set forth in Figures 2A-2G; and a nucleotide sequence which encodes a polypeptide that is 90% or more identical to an amino acid sequence set forth in Figures 2A-2G. The second *BRAF* nucleic acid may also be a

fragment of the foregoing comprising one or more polymorphic variations. The subject is often a human.

[00187] Gene therapy vectors can be delivered to a subject by, for example, intravenous injection, local administration (see U.S. Patent 5,328,470) or by stereotactic injection (see *e.g.*, Chen *et al.*, (1994) *Proc. Natl. Acad. Sci. USA 91*:3054-3057). Pharmaceutical preparations of gene therapy vectors can include a gene therapy vector in an acceptable diluent, or can comprise a slow release matrix in which the gene delivery vehicle is imbedded. Alternatively, where the complete gene delivery vector can be produced intact from recombinant cells (*e.g.*, retroviral vectors) the pharmaceutical preparation can include one or more cells which produce the gene delivery system. Examples of gene delivery vectors are described herein.

[00188] Pharmaceutical compositions can be included in a container, pack, or dispenser together with instructions for administration.

[00189] Pharmaceutical compositions of active ingredients can be administered by any of the paths described herein for therapeutic and prophylactic methods for treating melanoma. With regard to both prophylactic and therapeutic methods of treatment, such treatments may be specifically tailored or modified, based on knowledge obtained from pharmacogenomic analyses described herein. As used herein, the term "treatment" is defined as the application or administration of a therapeutic agent to a patient, or application or administration of a therapeutic agent to an isolated tissue or cell line from a patient, who has a disease, a symptom of disease or a predisposition toward a disease, with the purpose to cure, heal, alleviate, relieve, alter, remedy, ameliorate, improve or affect the disease, the symptoms of disease or the predisposition toward disease. A therapeutic agent includes, but is not limited to, small molecules, peptides, antibodies, ribozymes and antisense oligonucleotides.

[00190] Administration of a prophylactic agent can occur prior to the manifestation of symptoms characteristic of the BRAF aberrance, such that a disease or disorder is prevented or, alternatively, delayed in its progression. Depending on the type of *BRAF* aberrance, for example, a *BRAF* molecule, *BRAF* agonist, or *BRAF* antagonist agent can be used for treating the subject. The appropriate agent can be determined based on screening assays described herein.

[00191] As discussed, successful treatment of *BRAF* disorders can be brought about by techniques that serve to inhibit the expression or activity of target gene products. For example, compounds (*e.g.*, an agent identified using an assays described above) that exhibit negative modulatory activity can be used to prevent and/or treat melanoma. Such molecules can include, but are not limited to peptides, phosphopeptides, small organic or inorganic molecules, or antibodies (including, for example, polyclonal, monoclonal, humanized, anti-idiotypic, chimeric or single chain antibodies, and FAb, $F(ab')_2$ and FAb expression library fragments, scFV molecules, and epitope-binding fragments thereof).

[00192] Further, antisense and ribozyme molecules that inhibit expression of the target gene can also be used to reduce the level of target gene expression, thus effectively reducing the level of target gene activity. Still further, triple helix molecules can be utilized in reducing the level of target gene activity. Antisense, ribozyme and triple helix molecules are discussed above.

[00193] It is possible that the use of antisense, ribozyme, and/or triple helix molecules to reduce or inhibit mutant gene expression can also reduce or inhibit the transcription (triple helix) and/or translation (antisense, ribozyme) of mRNA produced by normal target gene alleles, such that the concentration of normal target gene product present can be lower than is necessary for a normal phenotype. In such cases, nucleic acid molecules that encode and express target gene polypeptides exhibiting normal target gene activity can be introduced into cells via gene therapy method. Alternatively, in instances in that the target gene encodes an extracellular polypeptide, it can be preferable to co-administer normal target gene polypeptide into the cell or tissue in order to maintain the requisite level of cellular or tissue target gene activity.

[00194] Another method by which nucleic acid molecules may be utilized in treating or preventing a disease characterized by *BRAF* expression is through the use of aptamer molecules specific for *BRAF* polypeptide. Aptamers are nucleic acid molecules having a tertiary structure which permits them to specifically bind to polypeptide ligands (see, *e.g.*, Osborne, *et al., Curr. Opin. Chem. Biol.1(1):* 5-9 (1997); and Patel, D. J., *Curr. Opin. Chem. Biol. Jun;1(1):* 32-46 (1997)). Since nucleic acid molecules may in many cases be more conveniently introduced into target cells than therapeutic polypeptide molecules may be, aptamers offer a method by which *BRAF* polypeptide activity may be specifically decreased without the introduction of drugs or other molecules which may have pluripotent effects.

[00195] Antibodies can be generated that are both specific for target gene product and that reduce target gene product activity. Such antibodies may, therefore, by administered in instances whereby negative modulatory techniques are appropriate for the treatment of *BRAF* disorders. For a description of antibodies, see the Antibody section above.

[00196] In circumstances where injection of an animal or a human subject with a *BRAF* polypeptide or epitope for stimulating antibody production is harmful to the subject, it is possible to generate an immune response against *BRAF* through the use of anti-idiotypic antibodies (see, for example, Herlyn, D., *Ann. Med.;31(1):* 66-78 (1999); and Bhattacharya-Chatterjee & Foon, *Cancer Treat. Res.; 94:* 51-68 (1998)). If an anti-idiotypic antibody is introduced into a mammal or human subject, it should stimulate the production of anti-anti-idiotypic antibodies, which should be specific to the *BRAF* polypeptide. Vaccines directed to a disease characterized by *BRAF* expression may also be generated in this fashion.

[00197] In instances where the target antigen is intracellular and whole antibodies are used, internalizing antibodies often are utilized. Lipofectin or liposomes can be used to deliver the antibody

or a fragment of the Fab region that binds to the target antigen into cells. Where fragments of the antibody are used, the smallest inhibitory fragment that binds to the target antigen often are utilized. For example, peptides having an amino acid sequence corresponding to the Fv region of the antibody can be used. Alternatively, single chain neutralizing antibodies that bind to intracellular target antigens can also be administered. Such single chain antibodies can be administered, for example, by expressing nucleotide sequences encoding single-chain antibodies within the target cell population (see *e.g.*, Marasco *et al., Proc. Natl. Acad. Sci. USA 90:* 7889-7893 (1993)).

[00198] *BRAF* molecules and compounds that inhibit target gene expression, synthesis and/or activity can be administered to a patient at therapeutically effective doses to prevent, treat or ameliorate *BRAF* disorders. A therapeutically effective dose refers to that amount of the compound sufficient to result in amelioration of symptoms of the disorders.

[00199] Toxicity and therapeutic efficacy of such compounds can be determined by standard pharmaceutical procedures in cell cultures or experimental animals, *e.g.*, for determining the LD_{50} (the dose lethal to 50% of the population) and the ED_{50} (the dose therapeutically effective in 50% of the population). The dose ratio between toxic and therapeutic effects is the therapeutic index and it can be expressed as the ratio LD_{50}/ED_{50}. Compounds that exhibit large therapeutic indices often are utilized. While compounds that exhibit toxic side effects can be used, care should be taken to design a delivery system that targets such compounds to the site of affected tissue in order to minimize potential damage to uninfected cells and, thereby, reduce side effects.

[00200] Data obtained from cell culture assays and animal studies can be used in formulating a range of dosage for use in humans. The dosage of such compounds lies preferably within a range of circulating concentrations that include the ED_{50} with little or no toxicity. The dosage can vary within this range depending upon the dosage form employed and the route of administration utilized. For any compound used, the therapeutically effective dose can be estimated initially from cell culture assays. A dose can be formulated in animal models to achieve a circulating plasma concentration range that includes the IC_{50} (*i.e.*, the concentration of the test compound that achieves a half-maximal inhibition of symptoms) as determined in cell culture. Such information can be used to more accurately determine useful doses in humans. Levels in plasma can be measured, for example, by high performance liquid chromatography.

[00201] Another example of effective dose determination for an individual is the ability to directly assay levels of "free" and "bound" compound in the serum of the test subject. Such assays may utilize antibody mimics and/or "biosensors" that have been created through molecular imprinting techniques. The compound which is able to modulate *BRAF* activity is used as a template, or "imprinting molecule", to spatially organize polymerizable monomers prior to their polymerization with catalytic reagents. The subsequent removal of the imprinted molecule leaves a polymer matrix which contains a repeated "negative image" of the compound and is able to selectively rebind the

molecule under biological assay conditions. A detailed review of this technique can be seen in Ansell *et al., Current Opinion in Biotechnology 7:* 89-94 (1996) and in Shea, *Trends in Polymer Science 2:* 166-173 (1994). Such "imprinted" affinity matrixes are amenable to ligand-binding assays, whereby the immobilized monoclonal antibody component is replaced by an appropriately imprinted matrix. An example of the use of such matrixes in this way can be seen in Vlatakis, *et al., Nature 361:* 645-647 (1993). Through the use of isotope-labeling, the "free" concentration of compound which modulates the expression or activity of *BRAF* can be readily monitored and used in calculations of IC_{50}. Such "imprinted" affinity matrixes can also be designed to include fluorescent groups whose photon-emitting properties measurably change upon local and selective binding of target compound. These changes can be readily assayed in real time using appropriate fiberoptic devices, in turn allowing the dose in a test subject to be quickly optimized based on its individual IC_{50}. A rudimentary example of such a "biosensor" is discussed in Kriz *et al., Analytical Chemistry 67:* 2142-2144 (1995).

[00202] Provided herein are methods of modulating *BRAF* expression or activity for therapeutic purposes. Accordingly, in an exemplary embodiment, the modulatory method involves contacting a cell with a *BRAF* or agent that modulates one or more of the activities of *BRAF* polypeptide activity associated with the cell. An agent that modulates *BRAF* polypeptide activity can be an agent as described herein, such as a nucleic acid or a polypeptide, a naturally-occurring target molecule of a *BRAF* polypeptide (*e.g.*, a *BRAF* substrate or receptor), a *BRAF* antibody, a *BRAF* agonist or antagonist, a peptidomimetic of a *BRAF* agonist or antagonist, or other small molecule.

[00203] In one embodiment, the agent stimulates one or more *BRAF* activities. Examples of such stimulatory agents include active *BRAF* polypeptide and a nucleic acid molecule encoding *BRAF*. In another embodiment, the agent inhibits one or more *BRAF* activities. Examples of such inhibitory agents include antisense *BRAF* nucleic acid molecules, anti-*BRAF* antibodies, and *BRAF* inhibitors. These modulatory methods can be performed *in vitro* (*e.g.*, by culturing the cell with the agent) or, alternatively, *in vivo* (*e.g.*, by administering the agent to a subject). As such, provided are methods of treating an individual afflicted with a disease or disorder characterized by aberrant or unwanted expression or activity of a *BRAF* polypeptide or nucleic acid molecule. In one embodiment, the method involves administering an agent (*e.g.*, an agent identified by a screening assay described herein), or combination of agents that modulates (*e.g.*, upregulates or downregulates) *BRAF* expression or activity. In another embodiment, the method involves administering a *BRAF* polypeptide or nucleic acid molecule as therapy to compensate for reduced, aberrant, or unwanted *BRAF* expression or activity.

[00204] Stimulation of *BRAF* activity is desirable in situations in which *BRAF* is abnormally downregulated and/or in which increased *BRAF* activity is likely to have a beneficial effect. For example, stimulation of *BRAF* activity is desirable in situations in which a *BRAF* is downregulated and/or in which increased *BRAF* activity is likely to have a beneficial effect. Likewise, inhibition of

BRAF activity is desirable in situations in which *BRAF* is abnormally upregulated and/or in which decreased *BRAF* activity is likely to have a beneficial effect.

[00205] The examples set forth below are intended to illustrate but not limit the invention.

Examples

[00206] In the following studies a group of subjects were selected according to specific parameters relating to melanoma. Nucleic acid samples obtained from individuals in the study group were subjected to genetic analysis, which identified associations between melanoma and certain polymorphic regions in the *BRAF* gene on chromosome seven. Methods are described for producing *BRAF* polypeptide and *BRAF* polypeptide variants *in vitro* or *in vivo*, *BRAF* nucleic acids or polypeptides and variants thereof are utilized for screening test molecules for those that interact with *BRAF* molecules. Test molecules identified as interactors with *BRAF* molecules and *BRAF* variants are further screened *in vivo* to determine whether they treat melanoma.

Example 1

Samples and Pooling Strategies

Sample Selection

[00207] Blood samples were collected from individuals diagnosed with melanoma, which were referred to case samples. Also, blood samples were collected from individuals not diagnosed with melanoma as gender and age-matched controls. A database was created that listed all phenotypic trait information gathered from individuals for each case and control sample. Genomic DNA was extracted from each of the blood samples for genetic analyses.

DNA Extraction from Blood Samples

[00208] Six to ten milliliters of whole blood was transferred to a 50 ml tube containing 27 ml of red cell lysis solution (RCL). The tube was inverted until the contents were mixed. Each tube was incubated for 10 minutes at room temperature and inverted once during the incubation. The tubes were then centrifuged for 20 minutes at 3000 x g and the supernatant was carefully poured off. 100-200 µl of residual liquid was left in the tube and was pipetted repeatedly to resuspend the pellet in the residual supernatant. White cell lysis solution (WCL) was added to the tube and pipetted repeatedly until completely mixed. While no incubation was normally required, the solution was incubated at 37°C or room temperature if cell clumps were visible after mixing until the solution was homogeneous. 2 ml of protein precipitation was added to the cell lysate. The mixtures were vortexed vigorously at high speed for 20 sec to mix the protein precipitation solution uniformly with the cell lysate, and then centrifuged for 10 minutes at 3000 x g. The supernatant containing the DNA was then poured into a clean 15 ml tube, which contained 7 ml of 100% isopropanol. The samples were

mixed by inverting the tubes gently until white threads of DNA were visible. Samples were centrifuged for 3 minutes at 2000 x g and the DNA was visible as a small white pellet. The supernatant was decanted and 5 ml of 70% ethanol was added to each tube. Each tube was inverted several times to wash the DNA pellet, and then centrifuged for 1 minute at 2000 x g. The ethanol was decanted and each tube was drained on clean absorbent paper. The DNA was dried in the tube by inversion for 10 minutes, and then 1000 µl of 1X TE was added. The size of each sample was estimated, and less TE buffer was added during the following DNA hydration step if the sample was smaller. The DNA was allowed to rehydrate overnight at room temperature, and DNA samples were stored at 2-8°C.

[00209] DNA was quantified by placing samples on a hematology mixer for at least 1 hour. DNA was serially diluted (typically 1:80, 1:160, 1:320, and 1:640 dilutions) so that it would be within the measurable range of standards. 125 µl of diluted DNA was transferred to a clear U-bottom microtitre plate, and 125 µl of 1X TE buffer was transferred into each well using a multichannel pipette. The DNA and 1X TE were mixed by repeated pipetting at least 15 times, and then the plates were sealed. 50 µl of diluted DNA was added to wells A5-H12 of a black flat bottom microtitre plate. Standards were inverted six times to mix them, and then 50 µl of 1X TE buffer was pipetted into well A1, 1000 ng/ml of standard was pipetted into well A2, 500 ng/ml of standard was pipetted into well A3, and 250 ng/ml of standard was pipetted into well A4. PicoGreen (Molecular Probes, Eugene, Oregon) was thawed and freshly diluted 1:200 according to the number of plates that were being measured. PicoGreen was vortexed and then 50µl was pipetted into all wells of the black plate with the diluted DNA. DNA and PicoGreen were mixed by pipetting repeatedly at least 10 times with the multichannel pipette. The plate was placed into a Fluoroskan Ascent Machine (microplate fluorometer produced by Labsystems) and the samples were allowed to incubate for 3 minutes before the machine was run using filter pairs 485 nm excitation and 538 nm emission wavelengths. Samples having measured DNA concentrations of greater than 450 ng/µl were re-measured for conformation. Samples having measured DNA concentrations of 20 ng/µl or less were re-measured for confirmation.

Pooling Strategies

[00210] Samples were placed into one of four groups, based on gender and disease status. The four groups were male case samples, male control samples, female case samples, and female control samples. A select set of samples from each group were utilized to generate pools, and one pool was created for each group. Each individual sample in a pool was represented by an equal amount of genomic DNA. For example, where 25 ng of genomic DNA was utilized in each PCR reaction and there were 200 individuals in each pool, each individual would provide 125 pg of genomic DNA. Inclusion or exclusion of samples for a pool was based upon the following criteria: the sample was

derived from an individual characterized as Caucasian; the sample was derived from an individual of German paternal and maternal descent; the database included relevant phenotype information for the individual; case samples were derived from individuals diagnosed with melanoma; control samples were derived from individuals free of cancer; and sufficient genomic DNA was extracted from each blood sample for all alleletyping and genotyping reactions performed during the study. Phenotype information included sex of the individual, number of nevi (few, moderate, numerous), hair color (black, brown, blond, red), diagnosed with melanoma (tumor thickness, date of primary diagnosis, age of individual as of primary diagnosis, post-operative tumor classification, presence of nodes, occurrence of metastases, subtype, location), country or origin of mother and father, presence of certain conditions for each individual (coronary heart disease, cardiomyopathy, arteriosclerosis, abnormal blood clotting/thrombosis, emphysema, asthma, diabetes type 1, diabetes type 2, Alzheimer's disease, epilepsy, schizophrenia, manic depression/bipolar disorder, autoimmune disease, thyroid disorder, and hypertension), presence of cancer in the donor individual or blood relative (melanoma, basaliom/spinaliom/lentigo malignant/mycosis fungoides, breast cancer, colon cancer, rectum cancer, lung cancer, lung cancer, bronchus cancer, prostate cancer, stomach cancer, leukemia, lymphoma, or other cancer in donor, donor parent, donor aunt or uncle, donor offspring or donor grandparent. Samples that met these criteria were added to appropriate pools based on gender and disease status.

[00211] The selection process yielded the pools set forth in Table 2, which were used in the studies that follow:

TABLE 2

	Male control	**Male case**	**Female control**	**Female case**
Pool size (Number)	217	236	233	266
Pool Criteria (ex: case/control)	control	case	control	case
Mean Age (ex: years)	48	51	47	49

Example 2

Association of Polymorphic Variants with Melanoma

[00212] A whole-genome screen was performed to identify particular SNPs associated with occurrence of melanoma. As described in Example 1, four sets of samples were utilized, which included samples from male individuals having melanoma (male melanoma cases), samples form individuals female individuals having melanoma (melanoma cases), samples from male individuals not having cancer (male controls), and samples from female individuals not having cancer (female

controls). The initial screen of each pool was performed in an alleletyping study, in which certain samples in each group were pooled. By pooling DNA from each group, an allele frequency for each SNP in each group was calculated. These allele frequencies were then compared to one another. Particular SNPs were considered as being associated with melanoma when allele frequency differences calculated between case and control pools, either male or female, were statistically significant. SNP disease association results obtained from the alleletyping study were then validated by genotyping each associated SNP across all samples from each pool. The results of the genotyping were then analyzed, allele frequencies for each group were calculated from the individual genotyping results, and a p value was calculated to determine whether the case and control groups had statistically significantly differences in allele frequencies for a particular SNP. When the genotyping results agreed with the original alleletyping results, the SNP disease association was considered validated at the genetic level.

[00213] It was discovered that four genotypes were associated with melanoma. In males and females, individuals having the haplotype CTTG corresponding to positions 146311, 138875, 76779, and 68398, respectively, in SEQ ID NO: 1 were predisposed to melanoma. Also, males having the haplotype ATGA corresponding to positions 146311, 138875, 76779, and 68398, respectively, in SEQ ID NO: 1, were predisposed to melanoma. In addition, males having an adenine at position 146311 of SEQ ID NO: 1, were predisposed to melanoma. Also, a male or female having the haplotype GGTTCGCATACT or GGTTCGTATATC, a female having the haplotype GATTCGCATACC, or a male having the haplotype TACCGATCCCTT (each twelve-position haplotype corresponds to positions 146311, 138875, 132526, 128002, 118712, 98846, 98682, 87826, 80400, 76779, 68398 and 64547, respectively, of SEQ ID NO: 1, and is reported in the forward orientation of the *BRAF* gene (complementary to the sequence in Figure 1)) were predisposed to melanoma.

SNP Panel Used for Genetic Analyses

[00214] A whole-genome SNP screen began with an initial screen of approximately 25,000 SNPs over each set of disease and control samples using a pooling approach. The pools studied in the screen are described in Example 1. The SNPs analyzed in this study were part of a set of 25,488 SNPs confirmed as being statistically polymorphic as each is characterized as having a minor allele frequency of greater than 10%. The SNPs in the set reside in genes or in close proximity to genes, and many reside in gene exons. Specifically, SNPs in the set are located in exons, introns, and within 5,000 base-pairs upstream of a transcription start site of a gene. In addition, SNPs were selected according to the following criteria: they are located in ESTs; they are located in Locuslink or Ensembl genes; and they are located in Genomatix promoter predictions. SNPs in the set were also selected on the basis of even spacing across the genome, as depicted in Table 2 and Figure 5.

TABLE 3

General Statistics		Spacing Statistics	
Total # of SNPs	25,488	Median	37,058 bp
# of Exonic SNPs	>4,335 (17%)	Minimum*	1,000 bp
# SNPs with refSNP ID	20,776 (81%)	Maximum*	3,000,000 bp
Gene Coverage	>10,000	Mean	122,412 bp
Chromosome Coverage	All	Std Deviation	373,325 bp
		Excludes outliers	

Alleletyping and Genotyping Results

[00215] The genetic studies summarized above and described in more detail below identified twelve allelic variants associated with melanoma, which are set forth in Table 4. Polymorphic variants and nucleic acids set forth in Tables 4, 7-10 and 12-16 correspond to the reverse orienation of the *BRAF* nucleotide sequence as it is set forth in Figure 1, while the polymorphic variants and nucleic acids set forth in Tables 17-21 are complementary to the reverse strand of the *BRAF* nucleotide sequence as it is set forth in Figure 1 (i.e., in the forward orientation).

TABLE 4

dbSNP rs#	Position in SEQ ID NO:1	Allele Variants
rs1639679	146311	A/C
rs1267646	138875	T/C
rs1267636	132526	A/G
rs1639675	128002	A/G
rs1267649	118712	G/C
rs1267609	98846	T/C
rs1267610	98682	A/G
rs1267625	87826	T/G
rs1267601	80400	G/A
rs1267606	76779	G/T
rs1267621	68398	A/G
rs1267618	64547	G/A

Assay for Verifying, Alleletyping, and Genotyping SNPs

[00216] A MassARRAY™ system (Sequenom, Inc.) was utilized to perform SNP genotyping in a high-throughput fashion. This genotyping platform was complemented by a homogeneous, single-tube assay method (hME™ or homogeneous MassEXTEND™ (Sequenom, Inc.)) in which two genotyping primers anneal to and amplify a genomic target surrounding a polymorphic site of interest. A third primer (the MassEXTEND™ primer), which is complementary to the amplified target up to but not including the polymorphism, was then enzymatically extended one or a few bases through the polymorphic site and then terminated.

[00217] For each polymorphism, SpectroDESIGNER™ software (Sequenom, Inc.) was used to generate a set of PCR primers and a MassEXTEND™ primer was used to genotype the polymorphism. Table 5 shows PCR primers and Table 6 shows extension primers used for analyzing polymorphisms. The initial PCR amplification reaction was performed in a 5 μl total volume containing 1X PCR buffer with 1.5 mM $MgCl_2$ (Qiagen), 200 μM each of dATP, dGTP, dCTP, dTTP (Gibco-BRL), 2.5 ng of genomic DNA, 0.1 units of HotStar DNA polymerase (Qiagen), and 200 nM each of forward and reverse PCR primers specific for the polymorphic region of interest.

TABLE 5

Reference SNP ID	Forward PCR primer	SEQ ID NO	Reverse PCR primer	SEQ ID NO
rs1639679	CTGAAACTGCAAGTAATGTTG		CCTACTTTTAAGCAAAATTCC	
rs1267646	TAAGCAGATTTTTGGTCCAG		GAAATGAAAGAAGTCATGGG	
rs1267636	GCACCAATGCTATTACTTGAG		TGGGAAACACATAGAGGCAG	
rs1639675	CAAGTACAAGGGAACACTTG		GCATAGAATTAGGACATGGC	
rs1267649	TTTTCATGAGCTGGACTCTG		CAAGGTTGAAGAGTAGGTTG	
rs1267609	GTTTGTCCAACTCAGAGATTG		GGGCTAACATTTACAAATGAC	
rs1267610	TTCATACTGCTTAACCTCTC		CCCAGCCAAAGAATGAATTAG	
rs1267625	GAGCAAGTACAGTTACTAGAC		TGGTAAGGAAATATGTTTGG	
rs1267601	ATTGCTACAAAGCAAGACAG		CAGAAACAGTGCAGAAAACAG	
rs1267606	CCACAAGTCCCCAAGATAAG		AGGACAGCATACATCAGACC	
rs1267621	ATCACAGTACTGAAAGCAAG		CTGTTTTTCAGGAATGTTCTG	
rs1267618	AGCATATGGTTCACATTGGC		TTTTCCAAAAGGATGGCCAC	

[00218] Samples were incubated at 95°C for 15 minutes, followed by 45 cycles of 95°C for 20 seconds, 56°C for 30 seconds, and 72°C for 1 minute, finishing with a 3 minute final extension at 72°C. Following amplification, shrimp alkaline phosphatase (SAP) (0.3 units in a 2 μl volume) (Amersham Pharmacia) was added to each reaction (total reaction volume was 7 μl) to remove any residual dNTPs that were not consumed in the PCR step. Samples were incubated for 20 minutes at 37°C, followed by 5 minutes at 85°C to denature the SAP.

[00219] Once the SAP reaction was complete, a primer extension reaction was initiated by adding a polymorphism-specific MassEXTEND™ primer cocktail to each sample. Each MassEXTEND™ cocktail included a specific combination of dideoxynucleotides (ddNTPs) and deoxynucleotides (dNTPs) used to distinguish polymorphic alleles from one another. In Table 6,

ddNTPs are shown and the fourth nucleotide not shown is the dNTP (*e.g.*, in the first row A, C and T are ddNTPs and G is the dNTP).

TABLE 6

Reference SNP ID	Position in SEQ ID NO:1	Extend Probe	Term Mix
rs1639679	146311	GTAATGTTGAAACTACAATTACCA	ACT
rs1267646	138875	GAAACAGGCTTCAATTCATCTT	ACT
rs1267636	132526	ACATAGAGGCAGGACTGTCA	ACT
rs1639675	128002	ATTAGGACATGGCTGAGATATTCA	ACT
rs1267649	118712	GGACTCTGCTTATTCTACCCA	ACT
rs1267609	98846	AGAGATTGTGCTTCCCAAATC	ACT
rs1267610	98682	GAATTAGTGAACTCTGGAAAGT	ACT
rs1267625	87826	GAAATATGTTTGGAAAATTGTTCT	ACT
rs1267601	80400	CTACAAAGCAAGACAGGACTAA	ACG
rs1267606	76779	CCAAGATAAGAATCTGTTTTACC	CGT
rs1267621	68398	AATGTTCTGAATTTTTCCAACTAA	ACT
rs1267618	64547	TTATAATTTAGTGGGGAACAGAA	ACG

[00220] The MassEXTEND™ reaction was performed in a total volume of 9 µl, with the addition of 1X ThermoSequenase buffer, 0.576 units of ThermoSequenase (Amersham Pharmacia), 600 nM MassEXTEND™ primer, 2 mM of ddATP and/or ddCTP and/or ddGTP and/or ddTTP, and 2 mM of dATP or dCTP or dGTP or dTTP. The deoxy nucleotide (dNTP) used in the assay normally was complementary to the nucleotide at the polymorphic site in the amplicon. Samples were incubated at 94°C for 2 minutes, followed by 55 cycles of 5 seconds at 94°C, 5 seconds at 52°C, and 5 seconds at 72°C.

[00221] Following incubation, samples were desalted by adding 16 µl of water (total reaction volume was 25 µl), 3 mg of SpectroCLEAN™ sample cleaning beads (Sequenom, Inc.) and allowed to incubate for 3 minutes with rotation. Samples were then robotically dispensed using a piezoelectric dispensing device (SpectroJET™ (Sequenom, Inc.)) onto either 96-spot or 384-spot silicon chips containing a matrix that crystallized each sample (SpectroCHIP™ (Sequenom, Inc.)). Subsequently, MALDI-TOF mass spectrometry (Biflex and Autoflex MALDI-TOF mass spectrometers (Bruker Daltonics) can be used) and SpectroTYPER RT™ software (Sequenom, Inc.) were used to analyze and interpret the SNP genotype for each sample.

Genetic Analysis

[00222] Twelve polymorphic variations identified in the *BRAF* gene are represented by SEQ ID NO: 1 at positions 146311, 138875, 132526, 128002, 118712, 98846, 98682, 87826, 80400, 76779, 68398 and 64547. Minor allelic frequencies for these polymorphisms was verified as being 10% or greater by determining the allelic frequencies using the extension assay described above in a group of samples isolated from 92 individuals originating from the state of Utah in the United States, Venezuela and France (Coriell cell repositories).

[00223] Table 7 shows alleletyping results in male melanoma and male control pools, and Table 8 shows alleletyping results in female melanoma and female control pools. Allele frequency is noted in the second and third columns for melanoma pools and control pools, respectively, and the allele indicated in bold type is the dominant allele. Genotyping results are shown for male pools in Table 9 and for female pools in Table 10. In the subsequent tables, "AF" refers to allelic frequency; "M case" and "M control" refer to male case and male control groups, respectively; and "F case" and "F control" refer to female case and female control groups, respectively.

TABLE 7

Position in SEQ ID NO:1	AF M case	AF M control	p-value
146311	**C**=0.695 A=0.305	*	*
138875	**C**=0.754 T=0.246	**C**=0.834 T=0.166	0.004
132526	**A**=0.875 G=0.125	**A**=0.913 G=0.087	0.074
128002	**A**=0.901 G=0.099	**A**=0.888 G=0.112	0.560
118712	**G**=0.875 C=0.125	**G**=0.927 C=0.073	0.011
98846	**C**=0.738 T=0.262	**C**=0.802 T=0.198	0.029
98682	**G**=0.584 A=0.416	**G**=0.577 A=0.423	0.846
87826	**T**=0.890 G=0.110	**T**=0.941 G=0.059	0.018
80400	**A**=0.801 G=0.199	**A**=0.871 G=0.129	0.006
76779	**T**=0.847 G=0.153	**T**=0.925 G=0.075	0.004
68398	**G**=0.712 A=0.288	**G**=0.840 A=0.160	0.000
64547	**G**=0.812 A=0.188	**G**=0.847 A=0.153	0.191

TABLE 8

Position in SEQ ID NO:1	AF F case	AF F control	p-value
146311	C=0.870 A=0.130	C=0.870 A=0.130	0.991
138875	C=0.804 T=0.196	C=0.830 T=0.170	0.315
132526	A=0.948 G=0.052	A=0.926 G=0.074	0.181
128002	A=0.955 G=0.045	A=0.948 G=0.052	0.688
118712	G=0.948 C=0.052	G=0.930 C=0.070	0.251
98846	C=0.851 T=0.149	C=0.830 T=0.170	0.383
98682	G=0.624 A=0.376	G=0.642 A=0.358	0.556
87826	T=0.905 G=0.095	T=0.895 G=0.105	0.651
80400	A=0.882 G=0.118	A=0.874 G=0.126	0.706
76779	T=0.927 G=0.073	T=0.929 G=0.071	0.935
68398	G=0.817 A=0.183	G=0.831 A=0.169	0.646
64547	G=0.861 A=0.139	G=0.849 A=0.151	0.610

TABLE 9

Position in SEQ ID NO:1	AF M case	AF M control	p-value
146311	C=0.888 A=0.112	C=0.927 A=0.073	0.045
138875	C=0.839 T=0.161	C=0.870 T=0.130	0.240
132526	A=0.891 G=0.109	A=0.939 G=0.061	0.010
128002	A=0.891 G=0.109	A=0.930 G=0.070	0.041
118712	G=0.896 C=0.104	G=0.934 C=0.066	0.038
98846	C=0.882 T=0.118	C=0.907 T=0.093	0.008
98682	G=0.732 A=0.268	G=0.770 A=0.230	0.187
87826	T=0.833 G=0.167	T=0.870 G=0.130	0.135
80400	A=0.894 G=0.106	A=0.932 G=0.068	0.041
76779	T=0.890 G=0.110	T=0.925 G=0.075	0.078

Position in SEQ	AF	AF	
68398	G=0.839 A=0.171	G=0.873 A=0.127	0.147
64547	G=0.837 A=0.163	G=0.871 A=0.129	0.146

TABLE 10

Position in SEQ ID NO:1	AF F case	AF F control	p-value
146311	C=0.943 A=0.057	C=0.934 A=0.066	0.585
138875	C=0.870 T=0.130	C=0.867 T=0.133	0.914
132526	A=0.945 G=0.055	A=0.935 G=0.065	0.517
128002	A=0.945 G=0.055	A=0.939 G=0.061	0.719
118712	G=0.945 C=0.055	G=0.934 C=0.066	0.474
98846	C=0.945 T=0.055	C=0.935 T=0.065	0.539
98682	G=0.766 A=0.234	G=0.784 A=0.216	0.507
87826	T=0.872 G=0.128	T=0.864 G=0.136	0.716
80400	A=0.944 G=0.056	A=0.935 G=0.065	0.526
76779	T=0.942 G=0.057	T=0.930 G=0.070	0.477
68398	G=0.868 A=0.132	G=0.880 A=0.120	0.584
64547	G=0.868 A=0.132	G=0.867 A=0.133	0.961

[00224] In Table 7, allelic frequency data for the male control group and the corresponding p-value for the SNP at position 146311 are not listed because the assay for this position failed. Results of the failed assay were supplemented by the genotyping data shown in Table 9. As can be seen in Table 9, alleles that included an adenine at position 146311 were enriched in the male melanoma group.

[00225] The extent of linkage disequilibrium (LD) between each pair of SNPs was estimated as the difference between the observed two locus haplotype frequency using the major alleles at each SNP and the product of the observed major allele frequencies. The disequilibrium between SNPs was also expressed by two other common standardized metrics, D' ($D/\min(p_1q_2, p_2q_1)$) and r^2 ($D^2/p_1p_2q_1q_2$), where p_1 and q_1 were the minor allele frequencies at two SNPs, and p_2 and q_2 were the corresponding major allele frequencies. Significant deviation of this disequilibrium from zero was tested by the use of a chi-square goodness-of-fit test.

[00226] The SNPs at positions 146311, 138875, 76779, and 68398 were found to be in strong LD by the D' and r^2 tests. Table 11 shows results of pairwise LD measurements using the D' method (results shown to the upper right of the diagonal) or the r^2 method (results shown to the lower left of the diagonal).

TABLE 11

	146311	138875	76779	68398
146311	1.0000	0.9675	0.9775	0.9759
138875	0.4364	1.0000	0.9519	0.9823
76779	0.9415	0.4287	1.0000	0.9604
68398	0.4812	0.8904	0.4730	1.0000

[00227] In view of the finding that the SNPs at positions 146311, 138875, 76779, and 68398 were in strong LD, haplotype analyses were carried out and the results are shown in Table 12. The number of individuals in the case or control pools having each haplotyped allele are set forth in Table 13, and the number of females or males having each haplotype are sub-categorized in Tables 14 and 15, respectively.

TABLE 12

Haplotype	Nucleotide Position in SEQ ID NO: 1				Frequency
	146311	138875	76779	68398	
H1	C	C	T	G	0.843
H2	A	T	G	A	0.074
H3	C	T	T	A	0.064
H4	C	T	T	G	0.012
H5	C	C	G	G	0.003
H6	A	C	T	G	0.002
H7	C	C	T	A	0.002
H8	A	C	G	A	0.001

TABLE 13

Haplotype	Case (N=1000)	Control (N=898)	Test statistic
CTTG	2%(20)	0%(3)	Chi squared = 16.75, P = 0.019095
CTTA	6%(64)	6%(58)	
CCTG	84%(829)	86%(771)	
CCTA	0%(2)	0%(1)	
CCGG	0%(1)	0%(4)	
ATGA	8%(82)	7%(59)	
ACTG	0%(1)	0%(2)	
ACGA	0%(1)	0%(0)	

TABLE 14

Females	Case (N=530)	Control (N=464)	Test statistic
CTTG	2%(9)	0%(1)	Chi squared=7.82, P=0.25152
CTTA	8%(40)	7%(32)	
CCTG	85%(449)	86%(397)	
CCTA	0%(0)	0%(1)	
CCGG	0%(1)	0%(2)	
ATGA	6%(30)	6%(29)	
ACTG	0%(1)	0%(2)	

TABLE 15

Males	Case (N=470)	Control (N=434)	Test statistic
CTTG	2%(11)	0%(2)	Chi squared=15.85, P=0.014568
CTTA	5%(24)	6%(26)	
CCTG	81%(380)	86%(374)	
CCTA	0%(2)	0%(0)	
CCGG	0%(0)	0%(2)	
ATGA	11%(52)	7%(30)	
ACGA	0%(1)	0%(0)	

[00228] Tables 13, 14, and 15 compare differences between haplotype frequencies in case groups and control groups, and demonstrated that there were significant differences in haplotype frequency between the male case group and the male control group, and between the combined case group and the combined control group. The haplotype-based genotype data in Tables 13, 14, and 15 were analyzed using a standard chi-square test of independence. The test of independence compared frequencies observed within each cohort, to those expected based on the margins of each contingency table. For genotypes and allelotypes, two tests were carried out on each SNP. One was carried out using the genotype data provided by laboratory measurements, which sometimes included a significant amount of missing information. A second test was carried out by including the genotypes inferred by the haplotype reconstruction algorithm in cases where the laboratory failed to obtain a valid genotype.

[00229] Table 16 shows results of a melanoma association analysis of chi-square components. This analysis demonstrated that the haplotypes CTTG and ATGA were significantly associated with melanoma. The test determined whether each haplotype was present at a different frequency in case groups and control groups. Where the two values for a given haplotype summed to a value greater than $\chi_1^2(0.95) = 3.84$, then a 2 x 2 contingency table comparing that haplotype versus all other haplotypes was statistically significant at a nominal level of $\alpha = 0.05$. The haplotype CTTG provided the greatest contribution to the total χ^2 statistic, and was statistically significant in the 2 x 2 contrasts. In males, the haplotype ATGA also provided a large contribution to the total χ^2 statistic. The most common haplotype, CCTG, did not differ significantly among cases and controls in any group.

TABLE 16

Tbl-hatest-x2	Combined		Female		Male	
	Case	Control	Case	Control	Case	Control
CTTG	5.1300	5.71000	2.52000	2.8800	2.660	2.880
CTTA	0.0012	0.00134	0.06750	0.0771	0.153	0.166
CCTG	0.2320	0.25900	0.00965	0.0110	0.368	0.399
CCTA	0.1110	0.12400	0.53300	0.6090	0.887	0.960
CCGG	1.0100	1.13000	0.22500	0.2570	1.040	1.130
ATGA	0.8000	0.89100	0.06760	0.0773	2.060	2.230
ACTG	0.2130	0.23800	0.22500	0.2570		
ACGA	0.4250	0.47300			0.443	0.480

[00230] Additional haplotype analyses were carried out using all twelve polymorphic variations identified in the *BRAF* gene and the results are shown in Table 17. Each twelve-position haplotype reported in Table 17 corresponds to positions 146311, 138875, 132526, 128002, 118712, 98846, 98682, 87826, 80400, 76779, 68398 and 64547, respectively, of SEQ ID NO: 1, and is reported in the forward orientation of the *BRAF* gene (complementary to the sequence in Figure 1). The number of individuals in the case or control pools having each haplotyped allele are set forth in Table 18, and the number of females or males having each haplotype are sub-categorized in Tables 19 and 20, respectively.

[00231] Haplotypes were reconstructed from the SNP genotypes using the statistical method developed by Stephens *et al., American J. Human Genetics 68*: 978-989 (2001), and implemented in the PHASE computer program (version 1.0). This method reconstructed a haplotype for each genotyped individual. In cases where multiple SNPs were in high linkage disequilibrium, complete haplotypes were inferred even in subjects with only partial genotype information. This method resulted in providing both a haplotype and a complete genotype for all individuals. The PHASE program was run with 1000 iterations, each consisting of 100 steps through the Markov chain, after a burn-in period of 1000 iterations.

TABLE 17

hap-tbl	Haplotype	Freq	Rel.Freq	Cum.Rel.Freq
1	GGTTCGCATAC C	1408	0.745	0.745
2	GGTTCGTATAC C	176	0.093	0.838
3	TACCGATCCCTT	134	0.071	0.909
4	GATTCGTCTATT	125	0.066	0.975
5	GATTCGCATAC C	13	0.007	0.982
6	GGTTCGTCTACC	5	0.003	0.985

hap-tbl	Haplotype	Freq	Rel.Freq	Cum.Rel.Freq
7	GGTTCGCATACT	5	0.003	0.988
8	GGTTCACATACC	5	0.003	0.991
9	GGTTCGCCTACC	3	0.002	0.993
10	TGTTCGCATACC	2	0.001	0.994
11	TGTCGACACCCC	2	0.001	0.995
12	GGTTCGTATATC	2	0.001	0.996
13	GGTTCGCATATC	2	0.001	0.997
14	GGTTCGCACACC	2	0.001	0.998
15	TGCTCGCATACC	1	0.001	0.999
16	TGCCGATCCCTT	1	0.001	1.000
17	TACCGATCTCTT	1	0.001	1.001
18	GGTTCACATATC	1	0.001	1.002
19	GGTCCGCATACC	1	0.001	1.003
20	GATTCGTCTCCT	1	0.001	1.004

TABLE 18

Haplotype	Case ($N = 1004$)	Control ($N = 900$)	Test Statistic
GATTCGCATACC	1% (12)	0% (1)	Chi squared = 22.41; $P = 0.26439$
GATTCGTCTATT	7% (67)	7% (58)	
GATTCGTCTCCT	0% (0)	0% (1)	
GGTCCGCATACC	0% (1)	0% (0)	
GGTTCACATACC	0% (3)	0% (2)	
GGTTCACATATC	0% (1)	0% (0)	
GGTTCGCACACC	0% (1)	0% (1)	
GGTTCGCATACC	73% (724)	77% (684)	
GGTTCGCATACT	0% (3)	0% (2)	
GGTTCGCATATC	0% (1)	0% (1)	
GGTTCGCCTACC	0% (2)	0% (1)	
GGTTCGTATACC	10% (100)	9% (76)	
GGTTCGTATAT	0% (0)	0% (2)	

Haplotype	Case (*N* = 1004)	Control (*N* = 900)	Test Statistic
C			
GGTTCGTCTAC C	0% (2)	0% (3)	
TACCGATCCCT T	8% (77)	6% (57)	
TACCGATCTCT T	0% (1)	0% (0)	
TGCCGATCCCT T	0% (1)	0% (0)	
TGCTCGCATAC C	0% (1)	0% (0)	
TGTCGACACCC C	0% (1)	0% (1)	
TGTTCGCATAC C	0% (0)	0% (2)	

TABLE 19

Females	Case (*N*=532)	Control (*N*=466)	Test Statistic
GATTCGCATAC C	2% (8)	0% (0)	Chi squared = 17.75; *P*=0.038239[1]
GATTCGTCTATT	8% (41)	7% (32)	
GGTTCGCACAC C	0% (1)	0% (0)	
GGTTCGCATAC C	75% (396)	78% (362)	
GGTTCGCATAC T	0% (0)	0% (2)	
GGTTCGCATAT C	0% (0)	0%(1)	
GGTTCGTATAC C	10% (53)	7%(34)	
GGTTCGTATATC	0% (0)	0%(2)	
GGTTCGTCTACC	0% (0)	0%(1)	
TACCGATCCCTT	5% (29)	6%(30)	

TABLE 20

Males	Case (*N* = 472)	Control (*N* = 434)	Test Statistic
GATTCGCATACC	1% (4)	0% (1)	Chi squared = 19.63; *P* = 0.35414[1]
GATTCGTCTATT	6% (26)	6% (26)	
GATTCGTCTCCT	0% (0)	0% (1)	
GGTCCGCATACC	0% (1)	0% (0)	
GGTTCACATACC	1% (3)	0% (2)	
GGTTCACATATC	0% (1)	0% (0)	
GGTTCGCACACC	0% (0)	0% (1)	
GGTTCGCATACC	70% (328)	75% (322)	
GGTTCGCATACT	1% (3)	0% (0)	
GGTTCGCATATC	0% (1)	0% (0)	
GGTTCGCCTACC	0% (2)	0% (1)	

Males	Case (*N* = 472)	Control (*N* = 434)	Test Statistic
GGTTCGTATACC	10% (47)	10% (42)	
GGTTCGTCTACC	0% (2)	0% (2)	
TACCGATCCCTT	10% (48)	6% (27)	
TACCGATCTCTT	0% (1)	0% (0)	
TGCCGATCCCTT	0% (1)	0% (0)	
TGCTCGCATACC	0% (1)	0% (0)	
TGTCGACACCCC	0% (1)	0% (1)	
TGTTCGCATACC	0% (0)	0% (2)	

[00232] Tables 17-20 compare differences between haplotype frequencies in case groups and control groups, and demonstrated that there were significant differences in haplotype frequency between the female case group and the female control group. The haplotype-based genotype data in Tables 17-20 were analyzed using a standard chi-square test of independence. The test of independence compared frequencies observed within each cohort, to those expected based on the margins of each contingency table. Also, the haplotypes disclosed in Tables 17-20 correspond to the forward orientation of the *BRAF* gene, whereas the genotypes and haplotypes of Tables 4, 7-10 and 12-16 are disclosed in the reverse orientation as they appear in Figure 1.

[00233] To better understand the results of the haplotype analysis in Tables 18-20, the chi-square components shown in Table 21 were considered. If the two values for a given haplotype summed to greater than Chi squared (0.95) = 3.84, then a 2 x 2 contingency table comparing that haplotype versus all other haplotypes were statistically significant at a nominal level of alpha = 0.05. This anlysis demonstrated that in the combined sexes and in females, the haplotype GATTCGCATACC provides the greatest contribution to the total Chi squared statistic, and was statistically significant in the 2 x 2 contrasts. Additionally, the haplotypes GGTTCGCATACT and GGTTCGTATATC contributed substantially to the total Chi squared statistic, both of which were rather rare. In males, the haplotype TACCGATCCCTT also was significant. The most common haplotype, GGTTCGCATACC, did not differ significantly among cases and controls in any group.

TABLE 21

tbl-hatest-x2	Combined		Female		Male	
	Case	Control	Case	Control	Case	Control
GATTCGCATACC	3.84000	4.30000	3.290	3.740	0.73100	0.80300
GATTCGTCTATT	0.01500	0.01680	0.118	0.135	0.05430	0.05970
GATTCGTCTCCT	0.52800	0.59100			0.52300	0.57500
GGTCCGCATACC	0.42200	0.47200			0.43400	0.47700
GGTTCACATACC	0.04900	0.05490			0.05610	0.06160
GGTTCACATAT	0.42200	0.47200			0.43400	0.47700

tbl-hatest-x2	Combined		Female		Male	
	Case	Control	Case	Control	Case	Control
C						
GGTTCGCACAC C	0.00298	0.00333	0.411	0.468	0.52300	0.57500
GGTTCGCATAC C	0.51100	0.57100	0.138	0.157	0.43800	0.48000
GGTTCGCATAC T	0.04900	0.05490	1.060	1.210	1.30000	1.43000
GGTTCGCATAT C	0.00298	0.00333	0.532	0.606	0.43400	0.47700
GGTTCGCCTAC C	0.10900	0.12200			0.11800	0.12900
GGTTCGTATAC C	0.53700	0.60100	0.968	1.100	0.00376	0.00413
GGTTCGTATAT C	1.06000	1.18000	1.060	1.210		
GGTTCGTCTAC C	0.15500	0.17400	0.532	0.606	0.00418	0.00459
TACCGATCCCT T	0.55100	0.61600	0.184	0.209	1.95000	2.14000
TACCGATCTCT T	0.42200	0.47200			0.43400	0.47700
TGCCGATCCCT T	0.42200	0.47200			0.43400	0.47700
TGCTCGCATAC C	0.42200	0.47200			0.43400	0.47700
TGTCGACACCC C	0.00298	0.00333			0.00209	0.00229
TGTTCGCATAC C	1.06000	1.18000			1.05000	1.15000

Example 3

Screening for *BRAF* Inhibitors

[00234] The following is an exemplary assay for finding inhibitors of *BRAF*. There are assays known in the art for detecting *Raf* inhibitors. See, *e.g.*, Hall-Jackson CA et al. *Chem Biol.* 1999 Aug;6(8):559-68. Inhibitors are identified by screening a compound library with a single or multi step coupled *in vitro* kinase cascade assay that measures the activation of MKK1 (an immediate downstream target of *BRAF*) in the presence of *BRAF*. Cells are transfected, transiently or often stably with the reporter construct described in Roth et al. *Genomics* 2000 Feb 1;63(3):384-90. The cells often are chosen for minimal expression of endogenous *BRAF*, so that it can be externally introduced. Cells expressing the construct are co-transfected with a *BRAF* expression vector or with vector lacking the *BRAF* sequence (control). Both sets of cells are treated with the test compound and downstream target activity is measured.

Example 4

Inhibition of *BRAF* Gene Expression by Transfection of Specific siRNAs

[00235] RNAi-based gene inhibition is a rapid way to inhibit expression of *BRAF* in cultured cells. siRNA reagents were selectively designed to target *BRAF*. Algorithms useful for designing siRNA molecules specific for *BRAF* are disclosed at the http address www.dhramacon.com. siRNA molecules up to 21 nucleotides in length are utilized. Table 22 summarizes the features of the duplexes that may be used in the assays described herein, where the sequence of one strand is shown (the other strand is complementary). A non-homologous siRNA reagent is used as a negative control.

TABLE 22: Duplex 21-mer siRNAs used for cell transfection

siRNA	siRNA Target	Sequence Specificity (5' to 3')	SEQ ID NO:
BRAF_229	BRAF	ATATATCTGGAGGCCTATG	
BRAF_264	BRAF	GCTAGATGCACTCCAACAA	
BRAF_1216	BRAF	TTACCTGGCTCACTAACTA	
BRAF_1231	BRAF	ACTAACGTGAAAGCCTTAC	
Control	Luciferase GL2	CGTACGCGGAATACTTCGA	

[00236] The siRNAs are transfected in cell lines MCF-7 and T-47D using Lipofectamine™ 2000 reagent from Invitrogen, Corp. 2.5 μg or 5.0 μg of siRNA is mixed with 6.25 μl or 12.5 μl lipofectamine, respectively, and the mixture is added to cells grown in 6-well plates. Their inhibitory effects on *BRAF* gene expression are confirmed by precision expression analysis using MassARRAY (quantitativeRT-PCR hME), which is performed on RNA prepared from the transfected cells (*see* Chunming & Cantor, *PNAS* 100(6):3059-3064 (2003)). RNA is extracted from cells two days after transfection. RNA is extracted with a Trizole reagent as recommended by the manufacturer (Invitrogen, Corp.) followed by cDNA synthesis using SuperScript™ reverse transcriptase. The specificity of the RNAi effect is confirmed by transfecting siRNA with a control sequence described in Table 22.

Example 5

In Vitro Production of *BRAF* Polypeptides

[00237] *BRAF* cDNA is cloned into a pIVEX 2.3-MCS vector (Roche Biochem) using a directional cloning method. A *BRAF* cDNA insert is prepared using PCR with forward and reverse primers having 5' restriction site tags (in frame) and 5-6 additional nucleotides in addition to 3' gene-specific portions, the latter of which is typically about twenty to about twenty-five base pairs in length. A Sal I restriction site is introduced by the forward primer and a Sma I restriction site is introduced by the reverse primer. The ends of *BRAF* PCR products are cut with the corresponding restriction enzymes (*i.e.*, Sal I and Sma I) and the products are gel-purified. The pIVEX 2.3-MCS

vector is linearized using the same restriction enzymes, and the fragment with the correct sized fragment is isolated by gel-purification. Purified *BRAF* PCR product is ligated into the linearized pIVEX 2.3-MCS vector and *E. coli* cells transformed for plasmid amplification. The newly constructed expression vector is verified by restriction mapping and used for protein production.

[00238] *E. coli* lysate is reconstituted with 0.25 ml of Reconstitution Buffer, the Reaction Mix is reconstituted with 0.8 ml of Reconstitution Buffer; the Feeding Mix is reconstituted with 10.5 ml of Reconstitution Buffer; and the Energy Mix is reconstituted with 0.6 ml of Reconstitution Buffer. 0.5 ml of the Energy Mix was added to the Feeding Mix to obtain the Feeding Solution. 0.75 ml of Reaction Mix, 50 µl of Energy Mix, and 10 µg of the *BRAF* template DNA is added to the *E. coli* lysate.

[00239] Using the reaction device (Roche Biochem), 1 ml of the Reaction Solution is loaded into the reaction compartment. The reaction device is turned upside-down and 10 ml of the Feeding Solution is loaded into the feeding compartment. All lids are closed and the reaction device is loaded into the RTS500 instrument. The instrument is run at 30°C for 24 hours with a stir bar speed of 150 rpm. The pIVEX 2.3 MCS vector includes a nucleotide sequence that encodes six consecutive histidine amino acids on the C-terminal end of the *BRAF* polypeptide for the purpose of protein purification. *BRAF* polypeptide is purified by contacting the contents of reaction device with resin modified with Ni^{2+} ions. *BRAF* polypeptide is eluted from the resin with a solution containing free Ni^{2+} ions.

Example 6
Cellular Production of *BRAF* Polypeptides

[00240] *BRAF* nucleic acids are cloned into DNA plasmids having phage recombination cites and *BRAF* polypeptides and polypeptide variants are expressed therefrom in a variety of host cells. λ phage genomic DNA contains short sequences known as attP sites, and *E. coli* genomic DNA contains unique, short sequences known as attB sites. These regions share homology, allowing for integration of phage DNA into *E. coli* via directional, site-specific recombination using the phage protein Int and the *E. coli* protein IHF. Integration produces two new att sites, L and R, which flank the inserted prophage DNA. Phage excision from *E. coli* genomic DNA can also be accomplished using these two proteins with the addition of a second phage protein, Xis. DNA vectors have been produced where the integration/excision process is modified to allow for the directional integration or excision of a target DNA fragment into a backbone vector in a rapid *in vitro* reaction (Gateway™ Technology (Invitrogen, Inc.)).

[00241] A first step is to transfer the *BRAF* nucleic acid insert into a shuttle vector that contains attL sites surrounding the negative selection gene, ccdB (*e.g.* pENTER vector, Invitrogen, Inc.). This transfer process is accomplished by digesting the *BRAF* nucleic acid from a DNA vector used for

sequencing, and to ligate it into the multicloning site of the shuttle vector, which will place it between the two attL sites while removing the negative selection gene ccdB. A second method is to amplify the *BRAF* nucleic acid by the polymerase chain reaction (PCR) with primers containing attB sites. The amplified fragment then is integrated into the shuttle vector using Int and IHF. A third method is to utilize a topoisomerase-mediated process, in which the *BRAF* nucleic acid is amplified via PCR using gene-specific primers with the 5' upstream primer containing an additional CACC sequence (*e.g.*, TOPO® expression kit (Invitrogen, Inc.)). In conjunction with Topoisomerase I, the PCR amplified fragment can be cloned into the shuttle vector via the attL sites in the correct orientation.

[00242] Once the *BRAF* nucleic acid is transferred into the shuttle vector, it can be cloned into an expression vector having attR sites. Several vectors containing attR sites for expression of *BRAF* polypeptide as a native polypeptide, N-fusion polypeptide, and C-fusion polypeptides are commercially available (*e.g.*, pDEST (Invitrogen, Inc.)), and any vector can be converted into an expression vector for receiving a *BRAF* nucleic acid from the shuttle vector by introducing an insert having an attR site flanked by an antibiotic resistant gene for selection using the standard methods described above. Transfer of the *BRAF* nucleic acid from the shuttle vector is accomplished by directional recombination using Int, IHF, and Xis (LR clonase). Then the desired sequence can be transferred to an expression vector by carrying out a one hour incubation at room temperature with Int, IHF, and Xis, a ten minute incubation at 37°C with proteinase K, transforming bacteria and allowing expression for one hour, and then plating on selective media. Generally, 90% cloning efficiency is achieved by this method. Examples of expression vectors are pDEST 14 bacterial expression vector with att7 promoter, pDEST 15 bacterial expression vector with a T7 promoter and a N-terminal GST tag, pDEST 17 bacterial vector with a T7 promoter and a N-terminal polyhistidine affinity tag, and pDEST 12.2 mammalian expression vector with a CMV promoter and neo resistance gene. These expression vectors or others like them are transformed or transfected into cells for expression of the *BRAF* polypeptide or polypeptide variants. These expression vectors are often transfected, for example, into murine-transformed a adipocyte cell line 3T3-L1, (ATCC), human embryonic kidney cell line 293, and rat cardiomyocyte cell line H9C2.

[00243] Modifications may be made to the foregoing without departing from the basic aspects of the invention. Although the invention has been described in substantial detail with reference to one or more specific embodiments, those of skill in the art will recognize that changes may be made to the embodiments specifically disclosed in this application, yet these modifications and improvements are within the scope and spirit of the invention, as set forth in the claims which follow. All publications or patent documents cited in this specification are incorporated herein by reference as if each such publication or document was specifically and individually indicated to be incorporated herein by reference.

[00244] Citation of the above publications or documents is not intended as an admission that any of the foregoing is pertinent prior art, nor does it constitute any admission as to the contents or date of these publications or documents. U.S. patents and other publications referenced herein are hereby incorporated by reference.